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| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |             |                         |                            | Form Approved<br>OMB No. 0704-0188                |                                           |
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| 14. ABSTRACT"<br>Due to the lack of an early detection test, most cases of OVCA are detected at late stages when the 5-year survival rate is <10%. Transvaginal ultrasound (TVUS) imaging, the currently favored method, it cannot detect OVCA at early stage due to its limited resolution. Tumor related malignant nuclear transformation occurs early in tumor development and leads to the release of Nuclear Matrix Proteins (NMP) into the circulation. Tumor associated neoangiogenesis (TAN) follows malignant nuclear transformation. $\alpha v\beta 3$ -integrins and death receptor (DR) 6 are two markers of ovarian TAN expressed by neoangiogenic microvessels. DR6 is also secreted in serum. If the detection limit of TVUS imaging can be enhanced, $\alpha v\beta 3$ -integrins-expressing microvessels can be an in vivo imaging marker of ovarian TAN and may be used together with serum anti-NMP antibodies and DR6 to detect early stage OVCA. Our overall goal was to improve the TVUS detectability of ovarian tumors and ovarian TAN vessels at early stage by $\alpha v\beta 3$ -integrins-targeted contrast enhanced ultrasound (CE-US) molecular imaging. Overall results of the study suggest that CE-US molecular imaging targeting ovarian $\alpha v\beta 3$ -integrins-expressing microvessels enhanced the ability of TVUS to detect ovarian tumors and ovarian TAN at early stage in laying hen model of spontaneous OVCA. Changes in OVCA related CE-US imaging indices were associated with the elevation of serum DR6 levels. Serum prevalence of anti-NMP antibodies was associated with malignant nuclear transformation and can be detected from serum even before the tumors become detectable through targeted contrast enhanced TVUS imaging. |             |                         |                            |                                                   |                                           |
| 15. SUBJECT TERMS<br>Ovarian cancer, Early detection, Targeted ultrasound imaging, $\alpha v\beta 3$ -integrins, animal model                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |             |                         |                            |                                                   |                                           |
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## INTRODUCTION:

Ovarian cancer (OVCA) remains the most lethal gynecologic malignancy among women. Women with OVCA exhibit specific symptoms only in later stages (Stage III and IV) where the 5-year survival rate is <10% [1, 2]. In contrast, 80-90% OVCA patients detected in stage I respond to treatment[3]. Unfortunately, symptoms of OVCA at the early stage are non-specific and early detection (<25% cases) generally occurs by happenstance[3]. Moreover, the capacity to follow tumor development non-invasively, if it was detected, is non-existent. A valid animal model overcomes the numerous problems associated with clinical studies[4]. Transvaginal ultrasound (TVUS) scanning is the currently favored method for *in vivo* detection of ovarian abnormalities including OVCA. Diagnosis by TVUS scanning is based on the detection of ovarian morphological and vascular abnormalities associated with the disease. However, the detection limits of traditional TVUS do not allow for detection of microvessels at early stages of the disease. Malignant nuclear transformation and changes in nuclear matrix proteins (NMPs) as well as ovarian tumor associated neoangiogenesis (TAN) occur early in OVCA development. Malignant changes in the nucleus are associated with the shedding of NMPs into the circulation which elicits autoimmune responses through production of anti-NMP antibodies.  $\alpha_v\beta_3$ -integrins and death receptors (DR) 6 are markers of ovarian TAN and are overexpressed by ovarian tumor associated microvessels. In OVCA patients, DR-6 is shed into the circulation. Thus anti-NMP antibodies and DR6 represents potential circulatory markers of malignant ovarian transformation and ovarian TAN. Our long term goal is to improve the TVUS detectability of ovarian TAN vessels at the early stage by molecular targeted ( $\alpha_v\beta_3$ -integrins) contrast enhanced ultrasound (CE-US) imaging. Contrast agents are developed to improve the visualization of TAN vessels by TVUS scanning. We are using laying hens because of the difficulty in identifying and accessing patients with early stage OVCA.

## BODY: the research accomplishments associated with each task outlined in the approved Statement of Work.

Our overall hypothesis is that early stage OVCA lesions can be detected in the hen model using  $\alpha_v\beta_3$ -integrin targeted CE-US together with anti-NMP antibodies and serum levels of DR6. This hypothesis is being achieved by accomplishing following three specific aims:

*Aim 1: To determine whether CE-US using microbubbles targeted to  $\alpha_v\beta_3$ -integrins will identify hens with ovarian TAN at early stage of OVCA;*

*Aim 2: To examine whether anti-NMP antibodies appear in serum before ovarian TAN becomes detectable;*

*Aim 3: To determine whether CE-US indices and serum DR-6 levels established in Specific Aim1 will detect ovarian TAN at early stage OVCA in anti-NMP antibody positive hens.*

## Accomplishments reported in year-1 (2010-2011):

### Task 1. Molecular targeted contrast enhanced ultrasound (CE-US) imaging of hen ovarian tumor associated neo-angiogenesis (TAN)

#### 1a. Scanning of hens by $\alpha_v\beta_3$ -integrins targeted contrast enhanced molecular imaging

- 1)Á Gray scale scanning: Pre-contrast and post-contrast examination of hen ovaries and recording of still images and video clips.
- 2)Á Contrast parameters: Contrast parameters including time of arrival, features of contrast agent binding and wash-out of unbound contrast agents were examined.
- 3)Á Doppler ultrasound indices: ovarian vasculature of hens was examined by pre- and post-contrast injection by Doppler ultrasound imaging. Resistive (RI) and pulsatility indices (PI) were recorded.



- 4)Á Blood samples: Collected before the injection of contrast agent, sera were obtained and archived.
- 5)Á Determination of death receptor (DR6) levels: Serum DR-6 levels were determined by immunoassay.

**1b.** Following ultrasound scanning, all animals were euthanized and ovaries were collected and processed for paraffin, frozen and molecular biological studies.

**1c.** Pathology, molecular biology and biochemical study of hen samples:

- 1.Á Paraffin and frozen sections from all ovarian tissues blocks from all hens were made and representative sections were stained by hematoxylin & eosin for the diagnosis of tumor or non-tumor abnormalities.
- 2.Á Immunostaining of paraffin or frozen ovarian sections for DR-6 and  $\alpha_v\beta_3$ -integrins as well as immunoblotting of ovarian homogenates for the expression of DR-6.
- 3.Á Data analysis and establishment of indices for targeted CE-US imaging, RI & PI values and serum DR-6 levels detective of ovarian TAN at early stage OVCA with reference to histopathology and expression of tumor associated angiogenic markers. These indices are being used for the detection of early stage OVCA in subsequent Task (Specific Aim-2).

#### **Detail Report on the accomplishments:**

**Specific Aim 1:** CE-US scanning using microbubbles targeted to  $\alpha_v\beta_3$ -integrins and establishment of CE-US indices detective of ovarian TAN at early stage of OVCA.

**Animals:** A total of 140 White Leghorn hens (3 years old) with low egg laying rates (<125eggs/year) were selected from a flock of laying hens. In the initial study, 20 hens were selected and divided into two groups (10 hens each) and injected with non-targeted or  $\alpha_v\beta_3$ -integrins targeted microbubbles (Visistar® Integrin, Targeson Inc., La Jolla, CA) (described as contrast agent throughout the text) and the binding specificity of contrast agent was confirmed and a dose of 10uL/kg body weight was found optimum. In the subsequent study, age matched 100 hens with low egg laying rates and 20 hens with normally egg laying rates and reared under similar environment were selected for CE-US scanning with contrast agent. Blood from all hens were collected prior to CE-US scanning and sera were obtained and stored at -80°C until further use.

#### **CE-US $\alpha_v\beta_3$ -integrins targeted molecular imaging: Sonography and Image Analysis**

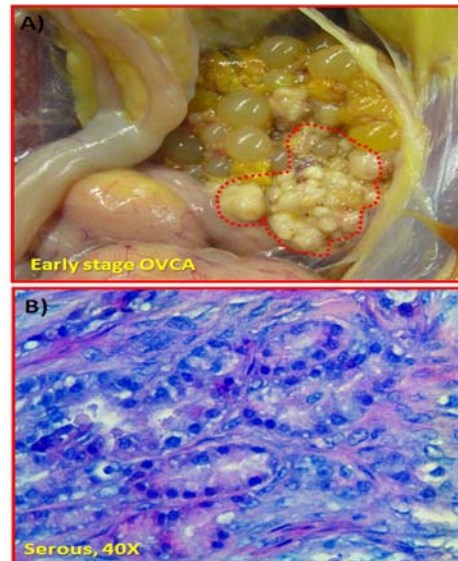
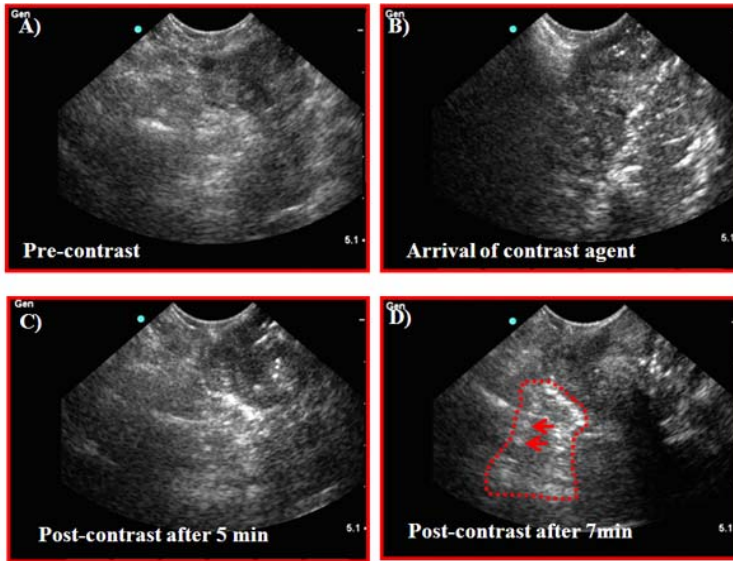
Pre-contrast scanning: Sonography was performed in a continuous pattern before and after the injection of contrast agent with the mechanical set up reported previously [4]. Briefly, all hens were scanned using an instrument equipped with a 5- to 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA). Each hen was immobilized, the transducer was inserted (transvaginal), and 2-dimensional transvaginal gray scale sonography as well as pulsed Doppler sonography was performed. The resistive index (RI: [systolic velocity – diastolic velocity]/systolic velocity) and the pulsatility index (PI: [systolic velocity – diastolic velocity]/mean) were automatically calculated from at least 2 separate images from the same ovary, and the lower RI and PI values were used for analysis. All images were processed and digitally archived.

#### Post-contrast Scanning

Post-contrast injection scanning was performed in a similar and continuous manner with identical mechanical settings as described above and the same pre-contrast imaged area was imaged according to the instruction of manufacturer of the contrast agent and earlier report [5]. Within 5-7 min from the arrival, contrast agent was accumulated at the target sites and unbound free microbubbles were washed out. All images were archived digitally in a still format as well as real-time clips (15 minutes for each hen). The effect of contrast agent was evaluated visually during the examination and afterward from reviewing the archived still images and video clips. The time of contrast agent arrival (interval in seconds from administration of the contrast agent to its visual observation [in seconds]) in the normal and tumor ovaries was recorded in real time. After review of the complete clip, the region of interest (ROI) was selected. The average image intensity over a ROI encompassing

the tumor was calculated and normalized by the pre-contrast intensity of the same ROI. The pixel intensity of ROI predictive of OVCA was determined. In addition, post-contrast RI and PI were calculated.

**Result:** Representative ultrasound images of an ovarian tumor at early stage before and after the injection of contrast agent are shown in **Figure 1** (B-mode, showing tumor size and borders. Normal ovaries in healthy hens with low egg laying rates (n = 20 selected hens) were found to have large one or two preovulatory and small growing stromal follicles on gray scale sonography. Blood vessels were detected in the ovarian stroma on color Doppler ultrasound imaging. Compared to pre-contrast scans, targeted imaging by contrast agent injection

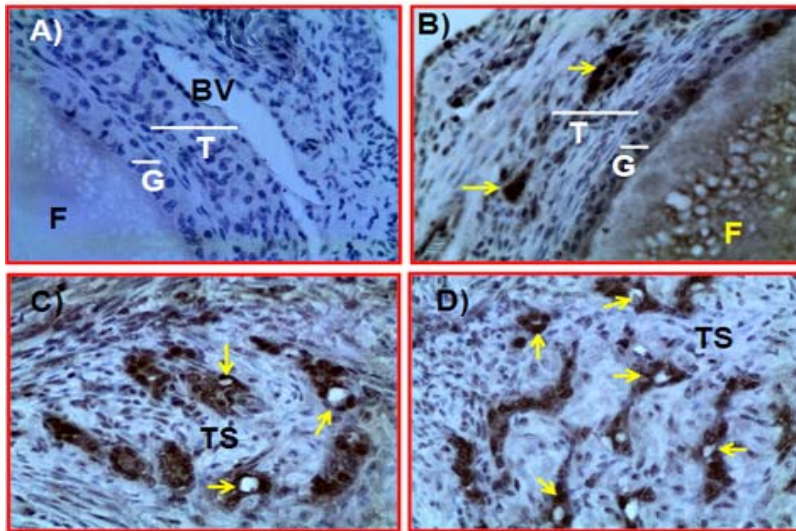


**Figure1.** Detection of ovarian tumors by  $\alpha_v\beta_3$ -integrins targeted contrast enhanced ultrasound (CE-US) molecular imaging. A) Pre-contrast gray scale ultrasound image of a hen ovary. Intensity of the signals from the tissue is very low and the presence of any solid mass is not decisive. B) Gray scale CE-US image of the same ovary at the arrival of contrast agent (CA). Compared to pre-contrast, remarkable increase in signal intensity is seen (appears like a white ring). But the binding of CA is not yet specific. C) Gray scale CE-US image of the same ovary 5 minutes after the injection of CA. With the washing out of unbound CA, bindings of CA are seen to be more specific. D) Gray scale CE-US image of the same ovary 10min after the injection of CA showing specific binding of CA. Regions of interest containing solid tissue mass bounded by targeted CA is indicated as red dotted circle. Arrows indicate the example of specific bindings of CA.

**Figure 2.** Ovarian tumor in laying hen detected by CE-US  $\alpha_v\beta_3$ -integrins targeted molecular imaging. A) Gross appearance of an ovarian tumor in a laying hen with stage I OVCA diagnosed in figure 1(D). Tumor was limited to a part of the ovary (red dotted line). B) Hematoxylin & eosin stained section of the corresponding ovarian tumor indicating the tumor is of serous histology. These observations confirmed the sonographic prediction that  $\alpha_v\beta_3$ -integrins targeted CE-US imaging can detect OVCA at early stage.

enhanced visualization of solid masses in the ovaries of 15 hens on gray scale and these hens were "predicted to have ovarian tumors" (**Figure 1D**). Overall, compared to pre-contrast, the pixel intensity of the signals from the ovary increased significantly after the injection of contrast agent. The signal intensity for low laying healthy hens was  $1651.89 \pm 563.79$  (mean  $\pm$  SD) pixels and

it was significantly higher in hens with a small solid ovarian mass predicted to have early stage OVCA ( $20,993.04 \pm 1621.44$ , range = 17983.38-23289.2, n = 7 hens) and late stage OVCA ( $25,130.35 \pm 953.73$  pixels) (n = 8 hens). The post-contrast RI and PI values for all of these hens with detectable solid ovarian mass were  $<0.40$  and  $<0.75$ , respectively. In addition, 9 hens were observed to have shrunken or regressed ovaries neither with preovulatory and small growing follicles nor with any detectable solid mass. Post-contrast RI and PI values of these hens ranged from 0.40-0.44 and 0.85-0.96, respectively and they were classified as hens with abnormal ovaries.



**Figure 3.** Immunohistochemical detection of DR-6 expressing microvessels by anti-chicken DR-6 antibodies (primary antibodies) in hen ovaries with or without tumor. A) Section of a normal ovarian stroma immunostained by omitting primary antibodies used as control. No immunopositive vessel is seen. B) Serial section from the same normal ovary immunostained with primary antibodies. Very few DR-6+ vessels are seen. C) Ovarian section of a hen with early stage ovarian cancer (OVCA). Compared to normal ovary, many DR-6+ microvessels are seen in the tumor stroma. D) Section of tumor ovary from a hen with late stage OVCA. Many DR-6+ microvessels are localized in the tumor stroma. BV= blood vessel, F= follicle, G= granulosa layer, T= theca layer, TS= tumor stroma. Arrows indicate the examples of DR-6+ microvessels. 40X.

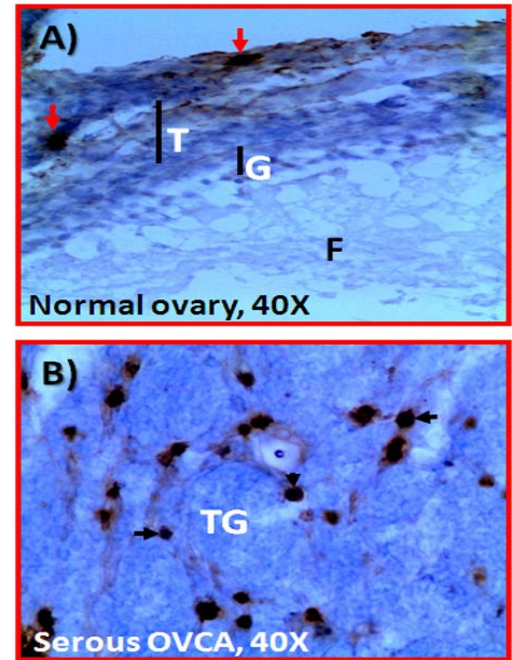
examination with hematoxylin-eosin staining (**Figure 2A-B**). As observed on sonography, late-stage OVCA (n = 8 hens: 4 serous, 3 endometrioid, 1 mucinous) was associated with moderate to profuse ascites and metastasis to distant organs. Early-stage ovarian tumor (n = 7 hens) was limited to the ovary (3 serous, 3 endometrioid, and 1 mucinous) with little or no ascites. Seven of 9 hens (2 serous, 3 endometrioid, 2 mucinous) initially classified as hens with abnormal ovarian morphologic characteristics without any grossly detectable solid ovarian masses during gray scale sonography had microscopic malignant ovarian lesions (hematoxylin-eosin staining) in 1 or more areas of the ovary and these hens were added to hens with early stage OVCA group. Thus the total number hens with early stage OVCA was (7 + 7) = 14.

#### Detection of Tumor associated neo-angiogenic (TAN) markers:

Sample preparation: Ovarian tissues from all hens including normal and tumor hens were processed for paraffin, frozen and molecular biological studies.

#### Ovarian Morphologic and histologic Evaluation:

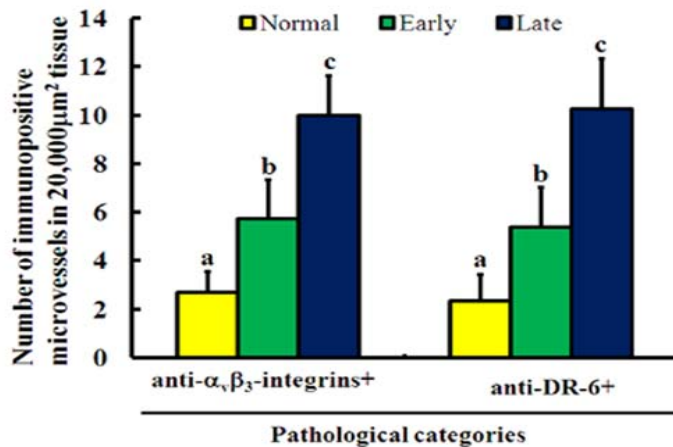
After CE-US molecular targeted imaging, all hens were euthanized and sonographic predictions were confirmed by gross examination of hens at necropsy. Ovarian tumors, their stages, and types were confirmed by routine histologic



**Figure 4:** Immunohistochemical detection of  $\alpha_v\beta_3$ -integrins expressing microvessels in hen ovaries predicted to be normal or to have ovarian tumor by molecular targeted CE-US imaging. A) Section of a normal hen ovary showing expression of few  $\alpha_v\beta_3$ -integrins stained vessels in the follicular theca and stroma beneath the ovarian surface. B) Ovarian section of a hen suspected to have ovarian tumor by molecular targeted CE-US imaging. In contrast to (A) many  $\alpha_v\beta_3$ -integrins expressing microvessels are seen in the tumor stroma. G, granulosa layer; T, theca layer of stromal follicle (F); TG, tumor gland. Arrows indicate the examples of  $\alpha_v\beta_3$ -integrins expressing microvessels.



**Detection of tissue expression of neo-angiogenic markers:** Paraffin and frozen sections were immunostained for the detection of DR-6+ and  $\alpha_v\beta_3$ -integrins+ microvessels and the frequencies of the immunopositive vessels were counted and analyzed. Differences in the frequency of immunopositive microvessels between normal and hens with OVCA were considered significant when the  $P < 0.05$ .



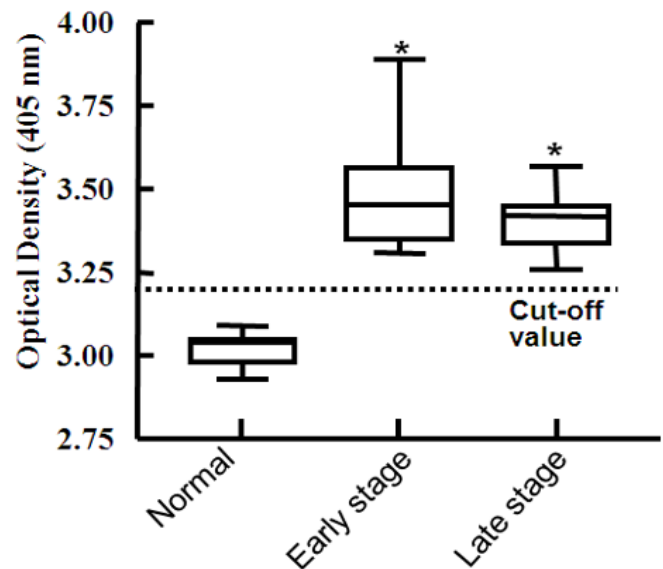
**Figure 5.** Changes in the frequency of tumor associated neo-angiogenic (TAN) markers (immunopositive ovarian  $\alpha_v\beta_3$ -integrins and DR-6 microvessels) relative to ovarian tumor development and progression in hens. Tissue expression of both the marker increased significantly in hens with early and late stages of OVCA. Detail information is included in the text. Each bar with different letter indicates significant differences ( $P < 0.05$ ) among different pathological groups.

seen in the follicular theca and the ovarian stroma. Compared to normal, many DR-6+ microvessels were localized in hens with OVCA (**Figure 3**). The population of DR-6+ microvessels was significantly ( $P < 0.05$ ) higher in hens with early stage OVCA (mean + SD= 5.38 + 1.64 in 20,000 $\mu\text{m}^2$  of tumor tissue) than that of normal hens (2.32 + 1.12 in 20,000 $\mu\text{m}^2$  of ovarian stromal tissue) and increased further in hens with late stage of OVCA (10.24 + 2.10 in 20,000 $\mu\text{m}^2$  of tumor tissue) (**Figure 5**).

**Tissue expression of  $\alpha_v\beta_3$ -integrins:** Similar to DR-6, microvessels expressing  $\alpha_v\beta_3$ -integrins were localized at the tumor vicinity (spaces between tumor glands) (**Figure 4**). Occasionally ovarian tumor epithelia also expressed  $\alpha_v\beta_3$ -integrins. Compared to normal hens (**Figure 4A**), the frequencies of  $\alpha_v\beta_3$ - integrin expressing microvessels were significantly ( $P < 0.05$ ) greater in hens with early stage OVCA (**Figure 4B**) and increased further in hens with late stage of OVCA. The population of  $\alpha_v\beta_3$ -integrins expressing microvessels in hens with early stage OVCA was significantly ( $P < 0.05$ ) higher (mean + SD= 5.72 + 1.61 in 20,000 $\mu\text{m}^2$  of tumor tissue) than that of normal hens (2.7 + 0.84 in 20,000 $\mu\text{m}^2$  of ovarian stromal tissue) and increased

**Result:** Neo-angiogenic immunopositive DR-6 or  $\alpha_v\beta_3$ -integrins expressing microvessels appeared to be leaky without any well organized continuous smooth muscle layer surrounding them.

**Tissue expression of DR-6:** In normal ovaries, very few immunopositive DR-6 expressing vessels were



**Figure 6.** Serum levels of death receptor (DR)-6 proteins in laying hens with or without (normal) ovarian cancer (OVCA) determined by immunoassay. Optical density (OD) values are displayed as a box-and-whiskers plot with median, range (whiskers), 25th to 75th percentile (box). DR-6 proteins in the sera of normal or OVCA hens were detected using rabbit anti-chicken DR-6 antibodies. Sera ( $n = 22$ ; age range 2.5 to 3.0 years) from hens with ovarian cancer were compared with normal hens (healthy hens of the same age,  $n = 50$  randomly selected from CE-US scanned hens). OD values of DR-6 greater than the cut off value (mean OD values + 3SD of normal hens) were considered as the diagnostic level of OVCA with reference to histopathology. All hens with OVCA pathology were found to have higher OD values for serum DR-6 levels than the diagnostic level of OVCA. \* indicates differences ( $P < 0.05$ ) than normal hens.

further in hens with late stage OVCA ( $9.97 \pm 1.64$  in  $20,000\mu\text{m}^2$  of tumor tissue) (**Figure 5**). These results confirm the observations of molecular targeted CE-US imaging that ovarian tumor associated microvessels express  $\alpha_v\beta_3$ -integrins which can be detected *in vivo* and may be used as imaging marker for the detection of OVCA at early stage.

**Serum Levels of DR-6:** DR-6 levels in the sera of normal or OVCA hens were determined by immunoassay using anti-chicken DR-6 antibodies and hens with optical density (OD) values for serum DR-6 level greater than a cut-off value (mean OD of normal hens + 3SD with reference to histopathology) was considered to have OVCA. The mean serum OD value for normal hens was (mean + SD =  $3.02 \pm 0.06$ ). The OD values for serum DR-6 levels in hens with early stage and late stage OVCA were greater than the mean OD + 3SD of normal hens. Hens with OD values for serum DR-6 levels greater than the cut-off value (mean + 3SD =  $3.02 \pm 0.06 \times 3 = 3.20$ ) were considered to have ovarian tumor associated neo-angiogenesis (TAN) (**Figure 6**).

Immunoreactivity of DR-6 proteins was confirmed by immunoblotting using selected sera samples.

**Tumor associated angiogenic indices (TANI):** Trends in the tumor associated changes in the frequencies of  $\alpha_v\beta_3$ -integrin and DR-6 expressing vessels were found similar in OVCA hens irrespective of tumor stages. A tumor associated neo-angiogenic index (TANI) diagnostic of OVCA with reference to histopathology was calculated as:  $\text{TANI} = \text{Ti} / \text{Ni} + \text{T}_d / \text{N}_d$ ; where  $\text{Ni}$  &  $\text{Ti}$  and  $\text{N}_d$  &  $\text{T}_d$  are the number of  $\alpha_v\beta_3$ -integrins and DR-6 positive vessels in normal and tumor hens, respectively. The value for neo-angiogenic index for normal ovary was considered as 2 (1 for  $\alpha_v\beta_3$ -integrins and 1 for DR-6 expressing vessels). TANI for early-stage OVCA was significantly higher than in hens with normal ovary (4.44 vs 2,  $P < 0.01$ , Mann-Whitney Exact test) and increased further as the disease progressed to late stage (8.11 vs 2,  $P < 0.01$ ). Moreover, TANI was inversely correlated to Doppler indices from OVCA hens (the higher the TANI, the lower the RI and PI) indicating that establishment of ovarian TAN is associated with increase in blood flow to the tumor vicinity. Taken together, the early stage OVCA diagnostic indices established with reference to histopathology and ovarian expression of TAN markers are:

- a. Pixel intensities diagnostic of early stage OVCA from molecular targeted CE-US imaging: 18000 pixels or greater
- b. Doppler indices diagnostic of early stage OVCA from molecular targeted CE-US imaging: RI = 0.44 or lower and PI = 0.96 or lower
- c. OD values for serum levels of DR-6 diagnostic of early stage OVCA = 3.20 or greater

## KEY RESEARCH ACCOMPLISHMENTS (Year-1):

- Established the enhancement of detectability of OVCA at early stage by traditional ultrasound imaging using by contrast enhanced  $\alpha_v\beta_3$ -integrins targeted molecular imaging.
- Confirmed the binding of contrast agents to the tissue marker of ovarian tumor associated neo-angiogenesis (microvessels expressing  $\alpha_v\beta_3$ -integrins) related to the early stage of OVCA.
- Ultrasound prediction of tumor associated overexpression of ovarian  $\alpha_v\beta_3$ -integrins confirmed by immunohistochemical detection.
- For the first time, OVCA diagnostic level of serum DR-6, a novel marker of tumor associated neo-angiogenesis at early stage was determined by an immunoassay.

- For the first time, by immunohistochemical detection, this study has shown that ovarian tumor as a source of serum DR-6 in this animal model.
- Establishment of the hypothesis that anti-NMP antibodies are associated with the early ovarian tumor formation is being underway.

## REPORTABLE OUTCOMES (Year-1):

**Presentation:** Abstract published and presented:

1. A. Barua, A. Yellapa, P. Bitterman, J. M. Bahr, S. Sharma, D. B. Hales, J. L. Luborsky, J. S. Abramowicz (2011): Use of contrast-enhanced ultrasound imaging with microbubbles targeted to  $\alpha_v\beta_3$  integrins to enhance detection of early-stage ovarian tumors, ASCO Annual Meeting. Chicago, IL, USA; June 3-7, 2011. (appended at pages 93-94)

[http://www.asco.org/ascov2/Meetings/Abstracts?&vmview=abst\\_detail\\_view&confID=102&abstractID=84636](http://www.asco.org/ascov2/Meetings/Abstracts?&vmview=abst_detail_view&confID=102&abstractID=84636)

2. A. Yellapa, JS. Abramowicz, P. Bitterman, JM. Bahr, MJ. Bradaric, SL. Edassery, S. Sharma, A. Barua. Interleukin (IL-16) and tumor associated neo-angiogenesis detects ovarian cancer at early stage. AACR 102th Annual Meeting, Orlando, FL, April 2-6, 2011. (Appended at page 95)

<http://www.abstractsonline.com/Plan/ViewAbstract.aspx?sKey=0732f136-3e77-422d-886b-29769face7da&cKey=fc49e373-cefe-430d-ae3f-058a41a93bf4&mKey=%7b507D311A-B6EC-436A-BD67-6D14ED39622C%7d>

## CONCLUSION (Aim-1): Year-1/Task-1

The results so far obtained with the accomplishment of Aim 1 suggest that contrast enhanced molecular imaging targeting ovarian  $\alpha_v\beta_3$ -integrins microvessels can detect ovarian tumor associated neo-angiogenesis at early stage of ovarian cancer in laying hen model of spontaneous ovarian cancer. Changes in early stage ovarian cancer related  $\alpha_v\beta_3$ -integrins-targeted CE-US imaging indices were associated with the elevation of serum levels of death receptor (DR-6).  $\alpha_v\beta_3$ -integrins-targeted CE-DUS imaging indices together with the serum levels of DR-6 detective of ovarian tumor associated neo-angiogenesis were used to diagnose hens with ovarian cancer at early stage in Aim 2.

## Accomplishments reported in year 2 of the project life (2011-2012):

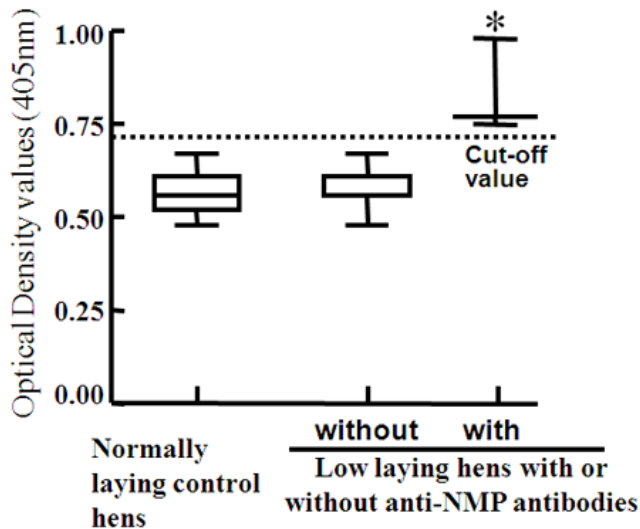
### Task 2. Appearance of anti-nuclear matrix protein (NMP) antibodies in serum and its association with ovarian TAN in prospective study.

**2a.** Hens with ovaries appearing normal (with normal egg laying rates and low egg laying rates) were scanned using indices established in Task 1 (specific Aim 1).

**2b.** Sera of all hens selected by CE-US scan (mentioned in 2a) were examined for the presence of anti-NMP antibodies by immunoassay and hens negative for serum anti-NMP antibodies or hens with normal levels of serum DR6 were selected for specific aim 2. These hens were monitored by CE-US scanning at 15 week intervals for the detection of ovarian TAN at early stage of OVCA. Sera from all hens were collected at each scan and analyzed for the appearance or prevalence of anti-NMP antibodies and DR6 levels.

**2c.** Hens suspected to have ovarian solid masses and/or ovarian TAN at each scan or all remaining hens at the end of the 45 week monitoring period were euthanized, examined at gross and tissue samples were processed for histopathology, immunohistochemistry including  $\alpha_v\beta_3$ -integrins and DR6 expression, proteomics and molecular biological examination.

**2d.** Correlation between serum prevalence of anti-NMP antibodies and DR6 levels with CE-DUS indices diagnostic of OVCA was examined.



**Figure 7.** Prevalence of anti-NMP antibodies in the sera of laying hens determined by immunoassay. Optical density (OD) values are displayed as a box-and-whiskers plot with median, range (whiskers), 25th to 75th percentile (box). Presence of anti-NMP antibodies in sera were examined in two assays using NMP antigens either from normal ovary or from ovarian tumors. Sera from hens of same age group (2.5 to 3.0 years old) with normal egg laying rates (n = 20, normal) and hens with low egg laying rates (n = 20) were used. Hens with OD values greater than the cut-off value (mean OD of normal + 3SD, dotted line) were considered positive for serum anti-NMP antibodies. At 1<sup>st</sup> CE-US targeted scan (after 15 weeks from the start), 3 out of 20 hens were found positive while 17 hens remain negative for serum anti-NMP antibodies. Differences in sera immunoreactivity was not observed between the antigens from normal or ovarian tumors.

after 15 weeks. All hens will be monitored for 45 weeks.

### **$\alpha_v\beta_3$ -integrins targeted contrast enhanced ultrasound (CE-US) imaging:**

Scanning of hens and analysis of sonograms were performed similar to *Specific Aim 1* which is briefly mentioned below: Pre-contrast scanning: Sonography was performed in a continuous pattern before and after the injection of contrast agent with the mechanical set up reported previously [7]. Briefly, all hens were scanned using an instrument equipped with a 5- to 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA). Each hen was immobilized, the transducer was inserted (transvaginally), and a 2-dimensional transvaginal gray scale sonography as well as a pulsed Doppler sonography was performed. The resistive index

### **Detail Report on the accomplishments for year 2 of the project life (2011-2012):**

***Specific Aim 2: Detection of Anti-NMP antibodies in the sera of laying hens and prospective monitoring of hens with anti-NMP antibodies to determine that anti-NMP antibodies appear in serum before ovarian TAN becomes detectable.***

A total of 40 laying hens with normally appearing ovaries (20 with low laying rates and without ovarian TAN and 20 with normal egg laying rates) were selected from a flock of laying hens by molecular targeted CE-US imaging using indices established in Aim 1 (mentioned above). Sera of all selected hens were examined for the presence of anti-NMP antibodies by immunoassay and hens negative for serum anti-NMP antibodies were selected for specific aim 2. Anti-NMP antibodies in the sera were determined by immunoassay using nuclear matrix protein extracts from normal or tumor ovaries as reported previously [6]. These hens are being monitored at 15 weeks intervals. Sera from all hens are being collected at each scan. At 1<sup>st</sup> scan (15 weeks from the start) sera samples of all hens were examined for anti-NMP antibodies.

**Results:** At first interval (after 15 weeks from the start of monitoring), 3 out of 20 hens in low egg laying group were found positive for sera anti-NMP antibodies (**Figure 7**). However, no abnormality in the ovarian morphology was detected in these hens at CE-US targeted imaging

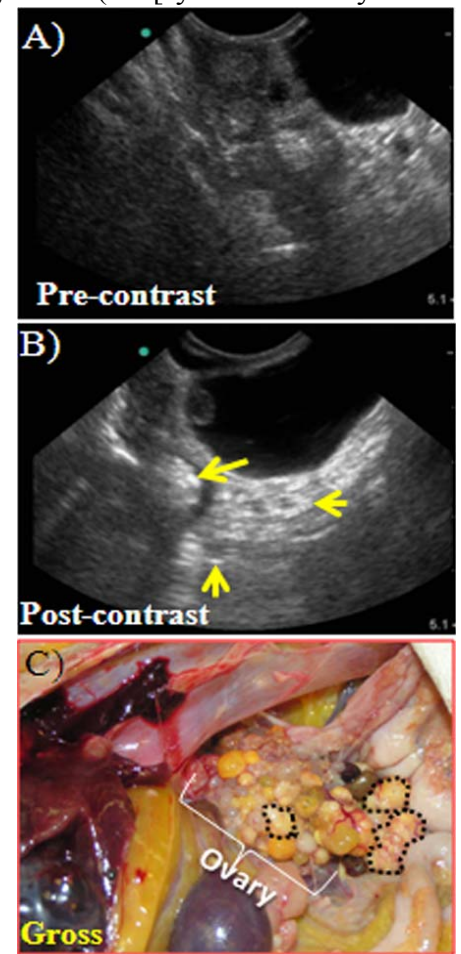


(RI: [systolic velocity – diastolic velocity]/systolic velocity) and the pulsatility index (PI: [systolic velocity – diastolic velocity]/mean) were automatically calculated from at least 2 separate images from the same ovary, and the lower RI and PI values were used for analysis. All images were processed and digitally archived.

#### Post-contrast Scanning

Post-contrast injection scanning was performed in a similar and continuous manner with identical mechanical settings as described above and the same pre-contrast imaged area was imaged according to the instruction of the manufacturer of the contrast agent and the earlier report [8]. Within 5-7 min from the arrival of the contrast agent, it accumulated at the target sites and unbound free microbubbles were washed out. All images were archived digitally in a still format as well as real-time clips (15 minutes for each hen). The effect of the contrast agent was evaluated visually during the examination and afterward from reviewing the archived still images and video clips. The time of the contrast agent arrival (interval in seconds from administration of the contrast agent to its visual observation [in seconds]) in the normal and tumor ovaries was recorded in real time. After review of the complete clip, the region of interest (ROI) was selected. The average image intensity over a ROI encompassing the tumor was calculated and normalized by the pre-contrast intensity of the same ROI. The pixel intensity of ROI predictive of OVCA was determined. In addition, post-contrast RI and PI were calculated.

**Result:** On gray scale sonography at initial scan, healthy hens with low egg laying rates ( $n = 20$  selected hens) showed one or two large preovulatory and several small growing follicles in the ovarian stroma. Blood vessels were detected in the ovarian stroma on color Doppler ultrasound imaging. Overall, similar to previous observation, compared with pre-contrast, the pixel intensity of the signals from the ovary increased significantly after the injection of the contrast agent. No detectable changes or abnormalities in ovarian morphology were observed in any of the experimental hens by targeted imaging at the 1<sup>st</sup> interval (15 weeks after initial scan). However, at the 2<sup>nd</sup> interval (30 weeks from initial scan) tumor related changes were suspected in 2 of 20 old hens with low egg laying rates. These morphological changes included a small solid tissue mass-like appearance limited to a part of the ovary with little or no ascitic fluid. Sonograms of these hens were processed and analyzed off-line immediately to determine the signal intensities in pixel values from the ROIs (consisting of the area suspected to have a solid mass) of the images. All of these hens had signal intensities above the pixel values indicative of tumor established in Specific Aim 1. These hens were euthanized and processed for subsequent tissue and molecular studies mentioned later. **Figure 8** shows representative ultrasound images of an ovary before and after the injection of  $\alpha_v\beta_3$ -integrins targeted contrast agent (B-mode, showing tumor size and borders). Post-contrast imaging predicted to have a small solid mass. At 3<sup>rd</sup> scan (45 weeks from the initial scan), 6 of the remaining 18 hens were diagnosed with ovarian tumor related changes. These 6 hens together with the remaining 12 hens with apparently normal ovaries were euthanized at the end of the 45 weeks. Thus, compared to pre-contrast scans, targeted imaging by  $\alpha_v\beta_3$ -integrins



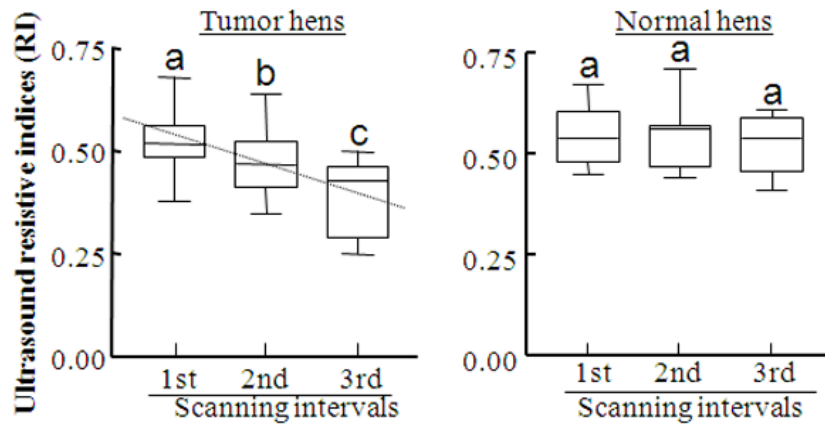
**Figure 8.**  $\alpha_v\beta_3$ -integrins-targeted molecular imaging probes enhanced the detection of spontaneous ovarian tumors in hens. A) Pre-contrast gray scale ultrasound image of a hen ovary showing low signal intensity from the tissue making the detection of any solid mass difficult. B) Post-contrast gray scale targeted imaging of the same ovary showing the enhancement of the ultrasound signal intensity indicative of solid tissue mass. Arrows indicate the examples of specific bindings of imaging agents with the targets. C) Gross appearance of ovarian tumor diagnosed at early stage in (B). Tumor masses are limited to a part of the ovary (black dotted circles).



targeted contrast agent enhanced visualization of solid masses in the ovaries of 8 hens on gray scale. All hens that developed ovarian tumors later showed a consistent decrease in RI and PI values even before the tumor become detectable (**Figure 9**). The post-contrast RI and PI values for all of these hens with detectable solid ovarian mass were <0.44 and <0.96, respectively as determined in Specific Aim 1. All hens with RI values less than 0.44 were predicted to have ovarian TAN based on their ovarian vascularity as depicted by Doppler ultrasound scan following the targeted imaging.

### Gross and histopathologic evaluation

All hens suspected of ovarian tumors and those remaining apparently normal during scanning by  $\alpha_v\beta_3$ -integrins



**Figure 9.** Changes in ultrasound resistive indices (RI) in association with ovarian tumor development in hens displayed as box and whiskers. The median, range (whiskers), and 25th to 75th percentiles (box) are shown. Hens were monitored prospectively by  $\alpha_v\beta_3$ -integrins targeted ultrasound imaging at 15 week intervals. RI values in hens (that developed tumors later) reduced consistently ( $P < 0.001$ ) from 1<sup>st</sup> scan to 3<sup>rd</sup> scan at the time of OVCA diagnosis (left panel, an imaginary dotted line shows the gradual reduction). Reduced RI values indicate lower resistance causing the increases flow of blood to the tissue, a characteristics feature associated with tumor development. Similar patterns of changes were also observed for PI values. However, such consistent changes in RI values were not observed in hens remaining normal. Bars with different letters indicate significant differences within the same group.

targeted contrast agent at different intervals including the final one at the end of the 45-weeks of prospective monitoring were euthanized. Gross ovarian morphology and stages of OVCA were recorded, normal ovaries and ovaries with tumor were harvested and processed for histopathological and immunohistochemical studies (paraffin and frozen), proteomic and molecular biological studies. Ovarian tumors, their stages, and types were confirmed by routine histologic examination with hematoxylin-eosin staining (**Figure 10A-B**). Predictions of targeted imaging were compared and confirmed by gross examination of hens at necropsy and from routine histology. As observed on sonography, ovarian tumors (n = 8 hens) were limited to the ovary (3 serous, 4 endometrioid, and 1 mucinous) with little or no ascites.

### Association of serum prevalence of anti-NMP antibodies and DR6 levels with the development and progression of ovarian tumors:

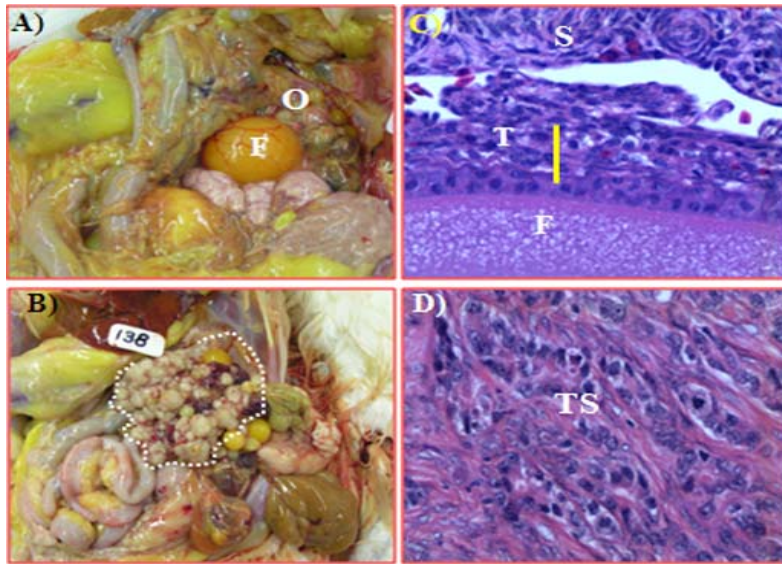
Blood from all hens was collected prior to ultrasound scanning and serum

samples in aliquots were stored at  $-80^{\circ}\text{C}$  until further use. Serum samples were used in immunoassay for the detection of anti-NMP antibodies and DR6 as well as in immunoproteomics for confirming the immunoreactivities of serum against the ovarian tumor NMPs.

**Prevalence of anti-NMP antibodies in serum:** The nucleus of the cell has long been used in the pathological diagnosis of cancer. Tumor associated changes in the molecular and morphological features of the nucleus represent the earliest event leading to malignant differentiation of the cell. Enlargement in size and irregular shape of the nucleus, re-organization of the chromatin and modifications in the composition of the nuclear matrix (which organizes the chromatin within the nucleus) are changes associated with malignant nuclear transformation. These morphological changes lead to the shedding or release of nuclear matrix proteins (NMPs) into the circulation eliciting an autoimmune response producing anti-NMP antibodies. Anti-NMP antibodies are

well established markers for several human autoimmune diseases and are associated with carcinoma of several organs.

Circulating anti-NMP antibodies were detected as reported previously with a little modification [6]. 96-well immunoassay plates were coated with tumor NMPs from hens with OVCA, overnight blocked and



**Figure 10.** Spontaneous ovarian tumors in laying hens diagnosed by  $\alpha_v\beta_3$ -integrins targeted ultrasound molecular imaging. A) A normal ovary in an old laying hen with low egg laying rates showing a preovulatory follicle in the ovary. B) An ovarian tumor at early stage in a laying hen. The tumor is limited to the ovary and appears like a cauliflower. Dotted line shows the tumor. C) Section of a normal ovary showing a growing follicle embedded in the ovarian stroma. D) Section of a serous ovarian carcinoma appearing like a sheet of malignant cells with pleomorphic nuclei surrounded by fibromuscular layer. F=follicle, O=ovary, S=stroma, T=theca layer, TS=tumor stroma. H&E, 40X.

incubated with serum from normal or hens with OVCA for two hours followed by rabbit anti-chicken alkaline phosphatase conjugated second antibodies for one hour.

Immunoreaction was developed using alkaline phosphates substrate and plates were read at 405nm and optical density (OD) values were recorded. 5 young laying hens with normal egg laying rates were used as controls. Mean OD + 3SD of control hens were used as cut-off values. Hens with OD values greater than the cut-off value were considered as positive for circulating anti-NMP antibodies. At the 1<sup>st</sup> interval (15 weeks after initial scan), 3 of 20 hens were found positive and additional 4 hens were positive at the 2<sup>nd</sup> interval (30 weeks after initial scan) and 4 of remaining 13 hens were positive for anti-NMP antibodies at the final scan (45<sup>th</sup> weeks after the initial scan). Thus a total of 11 of 20 hens had anti-NMP antibodies in their serum at the end of 45 week of monitoring. Once a hen became positive for anti-NMP antibodies it remained positive for subsequent intervals. All hens with OVCA were positive for serum anti-NMP antibodies. Immuno-proteomic analysis (two-dimensional Western blotting) using representative sera positive for anti-NMP antibodies showed

specific immunoreactivity of tumor sera against tumor NMPs (**Figure 11**).

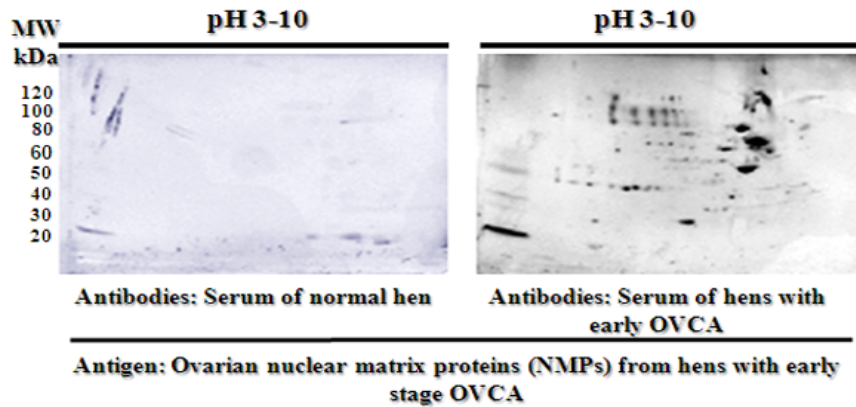
Changes in nuclear morphology are shown in **Figure 12**. Nuclear areas in normal or malignant cells were counted from 5 random fields at 100X in section stained with hematoxylin and eosin. The Mean (+SD) area (expressed in  $\mu\text{m}^2$ ) of the 5 largest nuclei were counted from a 100X light microscopic, filed and the mean of 5 fields were considered as the mean area of the nucleus in a normal or malignant ovarian section. The average of 3 sections from the same ovary was considered as the mean area of the nucleus of a hen with or without OVCA. 8 hens with OVCA (3 serous, 4 endometrioid and 1 mucinous) and 8 normal hens were used for nuclear morphometric analysis. As compared with the mean area of the nuclei in normal hens, the mean area of the nuclei was significantly greater in hens with OVCA or hens positive for serum anti-NMP antibodies (**Figure 13**). A nuclear morphological index (NMI) = nuclear area (NA) in (OVCA hens)/ (NA in control hens) was determined and a cut-off value ( $>2\text{X}$  NA of normal hens) was found diagnostic of OVCA based on tumor histopathology. These results suggest that malignant nuclear transformation is associated with the prevalence of anti-tumor antibodies in serum.

#### Association of DR6 levels with ovarian tumor development:

Death receptor (DR) 6 is a member of the tumor necrosis factor super family receptor associated with the induction of apoptosis under normal conditions. In contrast, apoptosis in ovarian tumors is reduced although DR6 expression is remarkably higher than in normal ovaries. DR6 is expressed in tumor cells and in the endothelium of tumor microvessels and is associated with malignant progression in human OVCA. DR6 has

been reported to be expressed in the laying hen ovary and shown to induce apoptosis in granulosa and theca cells of ovarian follicles. Importantly, the expression of DR6 by reproductive tissues that exhibit increased physiological angiogenesis, such as placenta, corpus luteum, or proliferative endometrium are significantly less as compared with tumor tissues.

DR6 levels in the sera of normal or OVCA hens were determined by immunoassay using anti-chicken DR6 antibodies. Hens with optical density (OD) values for serum DR6 level greater than a cut-off value established in Specific Aim 1 (mean OD of normal hens + 3SD,  $3.02 + 0.06 \times 3 = 3.20$ ) were considered to have OVCA. Hens with OD values for serum DR6 levels greater than the cut-off value were considered to have ovarian tumor associated neo-angiogenesis (TAN). All



**Figure 11.** Immunoproteomic detection of anti-NMP antibodies in serum of hens with or without OVCA. Membranes containing nuclear matrix proteins (NMPs) from ovarian tumor were reacted against normal and OVCA sera and immunostained for the detection of serum immunoreactivities for anti-NMP antibodies by 2dimensional Western blotting (2D-WB). Compared with serum from normal hens (left panel), serum from hens with OVCA reacted specifically against the ovarian NMPs from OVCA hens. The size of the reactive antigens mostly ranged from 30-80kD.

hens predicted to have ovarian tumors or ovarian TAN by the CE-DUS imaging had OD values for DR6 higher than the OVCA diagnostic level established in Aim 1 (mean + SD =  $3.02 + 0.06 \times 3 = 3.20$ ). Immunoreactivity of DR6 proteins was confirmed by immunoblotting using selected serum samples. These results suggest that the development of ovarian tumors is associated with an increase in serum levels of DR6.

#### **Tissue expression of markers of ovarian tumor associated neo-angiogenesis (TAN):**

Expression of  $\alpha_v\beta_3$ -integrins and DR6 by normal ovaries and ovaries with tumor was determined by immunohistochemistry using anti-DR6 and anti-  $\alpha_v\beta_3$  integrins antibodies. Immunopositive microvessels or cells were counted as reported previously [9].

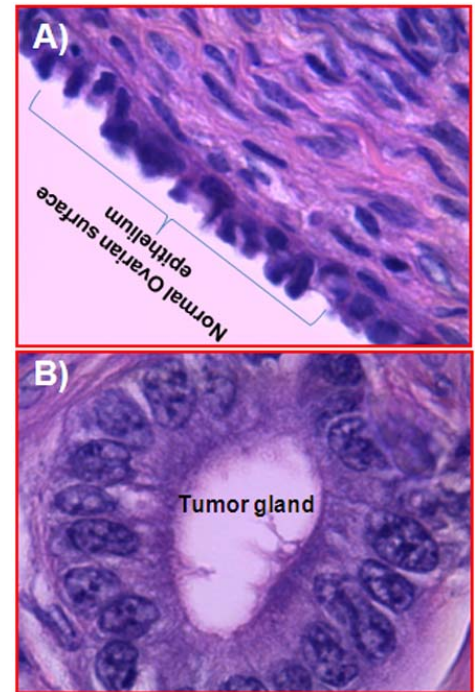
Expression of  $\alpha_v\beta_3$ -integrins:  $\alpha_v\beta_3$ -integrins were expressed by the endothelium of the immature and leaky angiogenic microvessels and localized at the tumor vicinity (spaces between tumor glands) (**Figure 14**) with occasional expression by ovarian epithelia. Compared to normal hens (**Figure 14A**), the frequencies of  $\alpha_v\beta_3$ -integrin expressing microvessels were significantly ( $P < 0.05$ ) greater in hens with early stage OVCA (mean + SD =  $5.84 + 1.66$  in  $20,000\mu\text{m}^2$  of tumor tissue) than normal hens ( $2.92 + 0.90$  in  $20,000\mu\text{m}^2$  of ovarian stromal tissue) (**Figure 14B**). -These results confirm that the greater signal intensities from hens with suspected ovarian tumor mass observed in the molecular targeted CE-US imaging suggest that ovarian tumor associated microvessels express  $\alpha_v\beta_3$ -integrins which can be detected *in vivo* and may be used as an imaging marker for the detection of OVCA at the early stage.



**Tissue expression of DR6:** Malignant ovarian tumor epithelium in OVCA hens as well as angiogenic microvessels was positive for DR6 expression (**Figure 15**). In normal ovaries, few microvessels in the follicular theca and in the stroma showed immunoreactivities for DR6 ( $2.81 \pm 0.56$  in  $20,000\mu\text{m}^2$  of ovarian stromal tissue) while many DR6+ microvessels were localized in hens with early stage OVCA (mean + SD=  $6.44 \pm 0.66$  in  $20,000\mu\text{m}^2$  of tumor tissue,  $P < 0.05$ ) (**Figure 16**).

Tumor associated neo-angiogenic index (TANI) diagnostic of OVCA established in Specific Aim 1 ( $\text{TANI} = \text{Ti} / \text{Ni} + \text{T}_d / \text{N}_d$ ; where  $\text{Ni}$  &  $\text{Ti}$  and  $\text{N}_d$  &  $\text{T}_d$  are the number of  $\alpha_v\beta_3$ -integrins and DR-6 positive vessels in normal and tumor hens, respectively) were evaluated for the detectability of ovarian TAN. TANI for normal ovary was considered as 2 (1 for  $\alpha_v\beta_3$ -integrins and 1 for DR-6 expressing vessels). Values of TANI from all hens at early stage OVCA were greater than normal hens ( $4.30$  vs  $2$ ,  $P < 0.01$ ).

As a whole, Specific Aim 2 showed that (1) serum anti-NMP antibodies appear before the tumor becomes detectable by targeted ultrasound imaging, positively correlated with the malignant nuclear transformation and ovarian TAN; (2) Serum prevalence of anti-NMP antibodies is correlated with the serum levels of DR6 detective of OVCA or ovarian TAN.



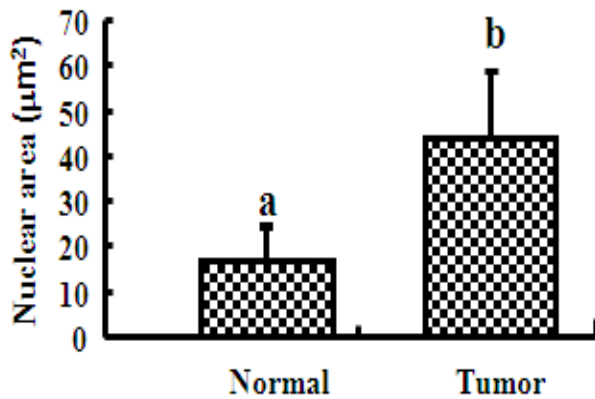
**Figure 12.** Changes in nuclear morphology of ovarian surface epithelial cells during malignant transformation. A) Section of a normal ovary showing ovarian surface epithelial cells with small and cuboidal or oval shaped nuclei. B) Ovarian section from a hen at early stage OVCA (containing a small tumor mass limited to a part of the ovary) and positive serum anti-NMP antibodies. Compared to normal, the nuclei increased in size and changed in shape (irregular) during malignant transformation. The nuclei become pleomorphic with mitotic bodies. H&E, 100X.

## KEY RESEARCH ACCOMPLISHMENTS (Year-2):

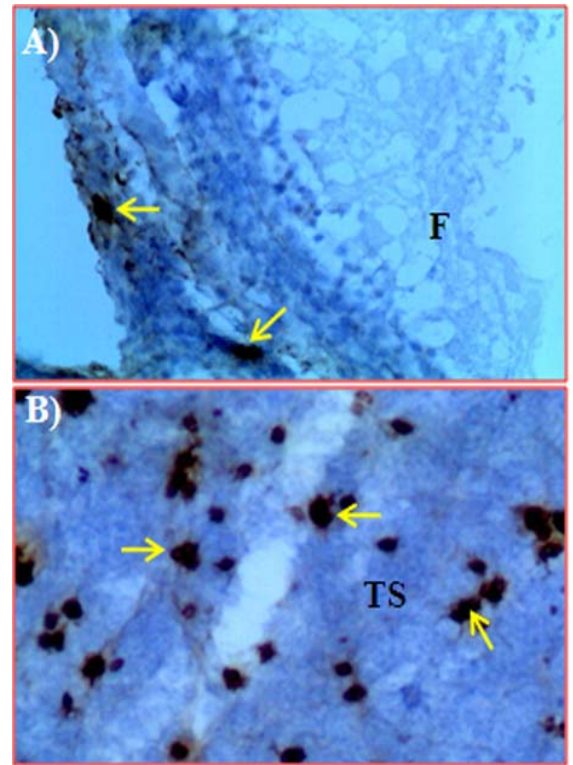
The key research accomplishments during the reporting period are:

- ❖ Established that ovarian tumor associated anti-NMP antibodies appear in serum before the tumor becomes detectable by targeted ultrasound imaging;
- ❖ Serum anti-NMP antibodies are an indicator of ovarian malignant nuclear transformation;
- ❖ Prevalence of serum anti-NMP antibodies is associated with the increase in serum DR6 levels detective of OVCA;
- ❖ Prevalence of serum anti-NMP antibodies is associated with changes in targeted ultrasound signal intensities relative to ovarian tumor development;
- ❖ Ovarian tumor associated neoangiogenic changes are associated with the prevalence of anti-NMP antibodies in serum;

- ❖ The indices for targeted ultrasound imaging diagnostic of OVCA including signal intensities, index for ovarian TAN and serum DR6 levels diagnostic of OVCA detected OVCA successfully.



**Figure 13.** Changes in nuclear size during malignant cellular transformation in the ovary of hens. Nuclear area is expressed as mean + SD in  $\mu\text{m}^2$  (n=8). Compared with normal ovarian surface epithelium, the nuclear area of malignant cells was significantly ( $P < 0.01$ ) greater in hens with early stage OVCA (n=8) than in normal hens. (n = 8). Bars with different letters indicate significant differences.



**Figure 14.** Immunohistochemical detection of  $\alpha_v\beta_3$ -integrins expressing microvessels in the stroma of a normal ovary or ovary with tumor at early stage in hens. Sections were immunostained with anti- $\alpha_v\beta_3$ -integrins antibodies. Compared with normal ovary (A), more  $\alpha_v\beta_3$ -integrins expressing microvessels are seen in the stroma of the ovaries with early stage OVCA (B). Arrows indicate the examples of immunopositive microvessels. F = stromal follicle, TS = tumor stroma.

## REPORTABLE OUTCOMES (Year-2):

### Manuscripts: Published

1. **Barua A**, Yellapa A, Bahr JM, Abramowicz JS, Edassery SL, Basu S, Rotmensch J and Bitterman P (2012). Expression of death receptor 6 (DR6) by ovarian tumors in laying hens, a preclinical model of spontaneous ovarian cancer, *Translational Oncology, Translational Oncology*, 2012; 5 (4), 260-268. PMID: 22937178  
<http://www.transonc.com/future.php?vol=5&no=4> (Appended at pages 68-76)
2. **Yellapa Y**, Bahr JM, Bitterman P, Abramowicz JS, Edassery SL, Penumatsa K, Basu S, Rotmensch J and **Barua A** (2012). Association of Interleukin 16 with the development of ovarian tumor and tumor associated neo-angiogenesis in laying hen model of spontaneous ovarian cancer *International Journal of Gynecological Cancer*, 22(2):199-207. PMID: [22274315](https://pubmed.ncbi.nlm.nih.gov/22274315/) (Appended at pages 77-85)

3. Khan M, Bahr JM, Yellapa Y, Bitterman P, Abramowicz JS, Edassery SL, Basu S, Rotmensch J and **Barua A (2012)**: Association of Immunoglobulin like transcript 3 (ILT3) with the progression of ovarian tumors in laying hens, a preclinical model of spontaneous ovarian cancer. *Translational Oncology*, 2012 April; 5(2): 85–91. PMID: [22496924](https://pubmed.ncbi.nlm.nih.gov/22496924/) (*Appended at pages 86-92*)

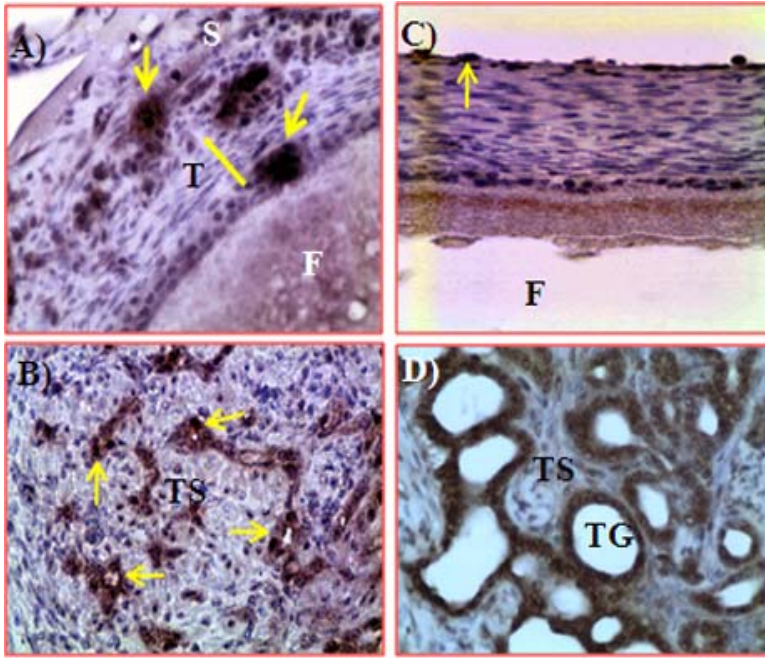
### **Presentations: Abstracts published and presented:**

1. Yellapa A, Khan MF, Bahr JM, Bitterman P, Abramowicz JS, Basu S, Edassery SL, **Barua A.** (2012). Expression of death receptor 6 (DR6) increases in association with ovarian tumor development and progression. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2012; 72(8 Suppl): Abstract nr 3646. doi:1538-7445.AM2012-3646 (*Appended at page 96*)  
[http://cancerres.aacrjournals.org/cgi/content/meeting\\_abstract/72/8\\_MeetingAbstracts/3646](http://cancerres.aacrjournals.org/cgi/content/meeting_abstract/72/8_MeetingAbstracts/3646)
2. Khan MF, Yellapa A, Bahr JM, Bitterman P, Abramowicz JS, Basu S, Edassery SL, **Barua A.** (2012). Expression of immunosuppressive leukocyte inhibitory immunoglobulin like transcript 3(ILT3) receptors increases with the development and progression of ovarian tumors. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2012; 72(8 Suppl): Abstract nr 5415. doi:1538-7445.AM2012-5415 (*Appended at pages 97*)  
[http://cancerres.aacrjournals.org/cgi/content/meeting\\_abstract/72/8\\_MeetingAbstracts/5415](http://cancerres.aacrjournals.org/cgi/content/meeting_abstract/72/8_MeetingAbstracts/5415)
3. **Barua A.** Molecular targeted ultrasound imaging of  $\alpha_v\beta_3$ -integrins expressing microvessels in association with anti-NMP antibodies detects ovarian cancer at early stage. **Oral presentation-symposium on Ovarian Cancer: Prevention, Detection, & Treatment of the Disease and Its Recurrence: Molecular mechanism and Personalized Medicine, held from May 10-11, 2012, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.** (*Appended at pages 98-99*)  
<http://www.upci.upmc.edu/ovarian/index.cfm>

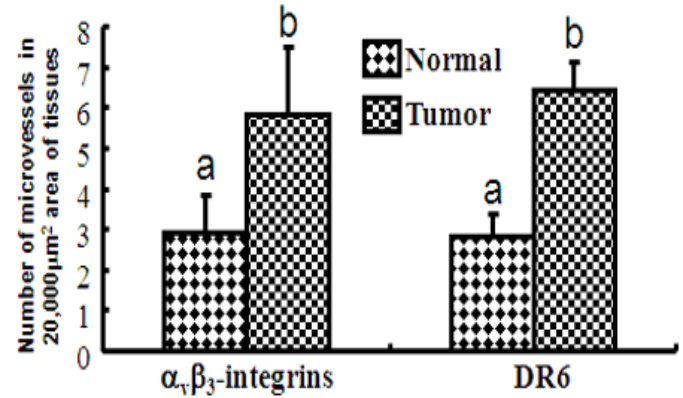
### **CONCLUSION (Aim-2): Year-2/Task-2**

The results of the accomplishments of Aim 1 and Aim 2 suggest that contrast enhanced molecular imaging targeting ovarian  $\alpha_v\beta_3$ -integrins can detect ovarian tumor associated neo-angiogenesis at early stage ovarian cancer in the laying hen model of spontaneous ovarian cancer. Changes in early stage ovarian cancer related CE-US imaging indices were associated with the elevation of serum levels of death receptor (DR6). Serum prevalence of anti-NMP antibodies is associated with tumor development and progression. Anti-NMP antibodies appear in serum before the tumor become detectable through imaging. Thus serum anti-NMP antibodies may be used as a surrogate marker of ovarian malignant nuclear transformation.





**Figure 15.** Immunohistochemical detection of DR6 expression in normal ovaries or ovaries with tumor. Sections were immunostained with anti- chicken DR6 antibodies. A- B) *DR6 expression by microvessels* in the stroma of a normal ovary (A) or ovary with tumor at early stage (B) in hens. Compared with normal ovary (A), more DR6 expressing microvessels are seen in the stroma of the ovaries with early stage OVCA (B). C-D) *DR6 expression by ovarian malignant epithelial cells*. Very few normal ovarian surface epithelium expressed DR6 (C). Compared with normal, strong immunoreaction was detected in malignant epithelium (D). Arrows indicate the examples of immunopositive microvessels or epithelial cells. F = stromal follicle, TG = tumor gland, TS = tumor stroma. 40X.



**Figure 16.** Changes in the frequencies of  $\alpha_v\beta_3$ -integrins and DR6 expressing microvessels in association with ovarian tumor development in hens. The numbers of immunopositive microvessels are expressed as mean + SD. Compared with normal hens (n = 11), the number of  $\alpha_v\beta_3$ -integrins and DR6 expressing microvessels was significantly ( $P < 0.01$ ) greater in OVCA hens (n= 8) than normal hens. Bars with different letters indicate significant differences within the same group.

### Accomplishments for the Year-3 (Year 3 of the project life, 2012-2013):

#### Task 3. Determination of specificity, sensitivity and predictive values of CE-DUS and serum markers diagnostic of OVCA (months 20-36).

- 3a.1. Hens were screened by  $\alpha_v\beta_3$ -integrins-targeted gray scale CE-US ultrasound imaging for the presence or absence of detectable ovarian mass using imaging indices established in specific Aim 1(Task 1). Serum DR6 levels and the prevalence of anti-NMP antibodies in these hens were examined similar to Tasks 1 and 2.
- 3a.2. Hens without any CE-US detectable ovarian solid mass with normal serum DR-6 levels (established in specific Aim 1) as well as with or without serum anti-NMP antibodies were selected (40 hens).

- 3b. Selected hens were monitoring for the development of ovarian tumor or TAN by  $\alpha_v\beta_3$ -integrins-targeted CE-US and Doppler ultrasound (DUS) scanning at 15-week intervals up to 45 weeks followed by final scan at 53 weeks (monitoring period was extended from originally proposed 45 weeks to 53 weeks to examine the incidence rates of OVCA in anti-NMP positive hens). Serum samples from all hens were collected at each scan and archived.
- 3c. Archived serum samples from CE-DUS scanning intervals were analyzed for DR-6 levels and prevalence of anti-NMP antibodies.
- 3d. Diagnosis of hens for suspected ovarian tumor or TAN: Once a hen showed targeted imaging indices established in specific Aim 1. It was predicted to have ovarian mass or ovarian TAN and euthanized following ultrasound diagnosis.
- 3e. Imaging indices and serum biochemical data including the presence of anti-NMP antibodies and the OVCA diagnostic levels of DR-6 established in specific Aim 1 were compared with the gross and microscopic presence of tumor. Detectability of early detection test established in specific Aim 1 and 2 (Tasks 1 and task 2) were determined.

**Specific Aim 3: CE-DUS imaging indices and serum DR-6 levels established in specific Aim 1 will detect ovarian tumor and ovarian TAN in anti-NMP antibody positive hens.** Aim 3 examined the detectability of early stage OVCA by  $\alpha_v\beta_3$ -integrins targeted CE-DUS imaging indices in association with serum DR-6 levels and anti-NMP antibodies established in Specific Aims 1 and 2.

Animals: 3-year old White Leghorn laying hens were scanned by  $\alpha_v\beta_3$ -integrins targeted CE-US imaging using indices established in Specific Aim 1 (RI >0.44 and PI > 0.96, pixel intensities <18000 pixels) and their sera were tested for the prevalence of anti-NMP antibodies and DR6 levels as reported earlier [5]. A total of 40 hens with low egg laying rates with (n=20) or without (n=20) serum anti-NMP antibodies and with normal DR-6 levels as well as without any ovarian mass (detectable by targeted CE-US imaging) were selected. Anti-NMP antibodies in the sera were determined by immunoassay using nuclear matrix protein (NMP) extracts from normal or tumor ovaries as reported previously [6]. Hens were monitored for 53 weeks by  $\alpha_v\beta_3$ -integrins targeted CE-US imaging at 15-week intervals up to 45 weeks followed by a final scan at 53 weeks. Sera from all hens were collected at each scan and stored at  $-80^{\circ}\text{C}$  until further use.

#### **Prospective monitoring of hens by $\alpha_v\beta_3$ -integrins-targeted CE-US imaging:**

Monitoring of hens by  $\alpha_v\beta_3$ -integrins-targeted CE-US and analysis of images were performed similar to *Specific Aim 1* which is briefly mentioned below:

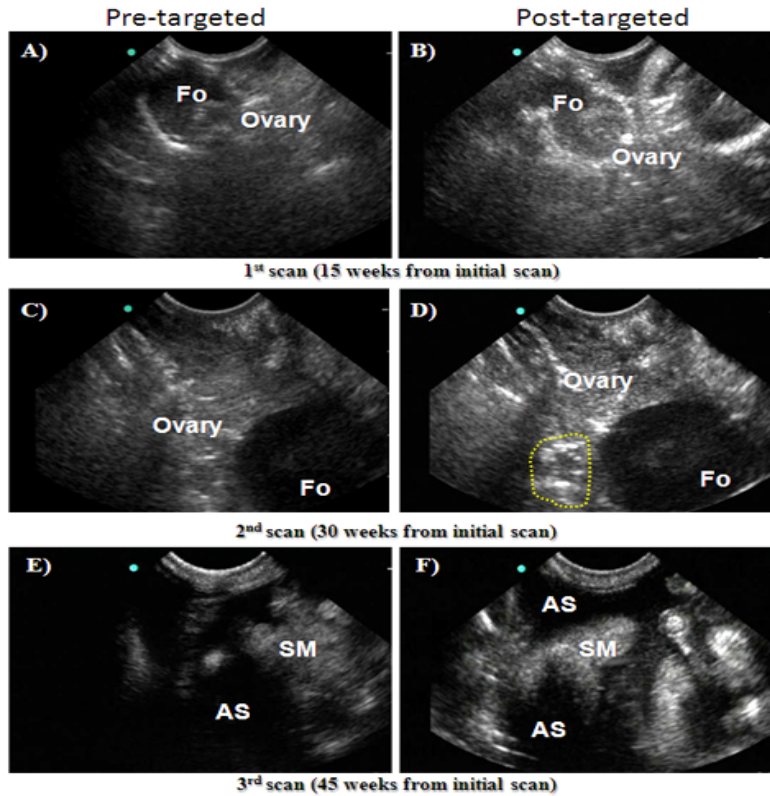
Pre-contrast scanning: Sonography was performed in a continuous pattern before and after the injection of contrast agent with the mechanical set up reported previously [7]. Briefly, all hens were scanned using an instrument equipped with a 5- to 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA). Each hen was immobilized, the transducer was inserted (transvaginally), and a 2-dimensional transvaginal gray scale sonography as well as a pulsed Doppler sonography was performed. The resistive index (RI: [systolic velocity – diastolic velocity]/systolic velocity) and the pulsatility index (PI: [systolic velocity – diastolic velocity]/mean) were automatically calculated from at least 2 separate images from the same ovary, and the lower RI and PI values were used for analysis. All images were processed and digitally archived.



## Post-contrast Scanning

Post-contrast injection scanning was performed in a similar and

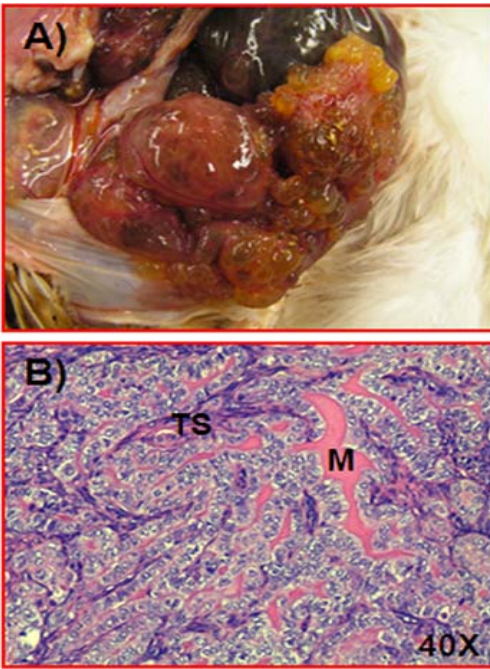
continuous manner with identical mechanical settings as described above and the same pre-contrast imaged area was imaged according to the instruction of the manufacturer of the contrast agent and the earlier report [8]. Within 5-7 min from the arrival of the contrast agent, it accumulated at the target sites and unbound free microbubbles were washed out. All images were archived digitally in a still format as well as real-time clips (15 minutes for each hen). The effect of the contrast agent was evaluated visually during the examination and afterward from reviewing the archived still images and video clips. The time of the contrast agent arrival (interval in seconds from administration of the contrast agent to its visual observation [in seconds]) in the normal and tumor ovaries was recorded in real time. After review of the complete clip, the region of interest (ROI) was selected. The average image intensity over a ROI encompassing the tumor was calculated and normalized by the pre-contrast intensity of the same ROI. The pixel intensity of ROI predictive of OVCA was determined. In addition, post-contrast RI and PI were calculated.



**Figure 17:** Development of ovarian tumor in a hen with serum anti-NMP antibodies monitored and detected by  $\alpha_v\beta_3$ -integrins-targeted contrast enhanced ultrasound imaging in a prospective study. The hen was monitored by targeted ultrasound scanning at 15-week intervals for 45 weeks. A-B) Pre- and post-targeted sonograms at first scan (15 weeks from the start of the study). The ovary was normal and had two preovulatory follicles (Fo). C-D) Sonograms of the same ovary at 2<sup>nd</sup> scan (30-weeks after the start of the study). The ovary had one preovulatory follicle, however, post-targeted imaging showed a small solid mass-like (SM, yellow dotted line) structure. E-F) Sonograms at 45-weeks (3<sup>rd</sup> scan) showing large solid ovarian masses accompanied with profuse ascites (AS) detected by targeted scanning. Thus,  $\alpha_v\beta_3$ -integrins-targeted imaging agent enhanced the ultrasound signal intensity for the detection of early stage ovarian tumors.

ovary increased significantly in post-contrast imaging following the injection of the contrast agent. Although 1 or 2 large preovulatory follicles were seen in a few hens, no pathology or detectable abnormalities in ovarian morphology were observed in any of these hens by targeted CE-US imaging at the 1<sup>st</sup> interval (15 weeks after initial scan) (**Figures 17A-B**). However, at the 2<sup>nd</sup> interval (30 weeks from initial scan) tumor associated changes in imaging indices (RI and PI values) as established in specific Aim 1 as well as morphological changes were observed in 6 of 20 hens with serum anti-NMP-antibodies-group. These tumor associated morphological changes included a small solid tissue mass in a part of the ovary accompanied with or without a little or moderate ascite like fluids. These hens were predicted to have OVCA and their sonograms were processed and analyzed off-line immediately to determine the signal intensities from the ROIs (consisting of the area suspected to have a solid

mass) of the images. All of these 6 hens had signal intensities above the pixel values indicative of early stage OVCA established in Specific Aim 1. These hens were euthanized; gross morphology was recorded and compared with targeted CE-US imaging. Serum samples were collected before the euthanasia and ovarian tissues processed for subsequent tissue and molecular studies mentioned later. However, 2 hens suspected to have a small solid



**Figure 18:** Confirmation of prediction of contrast enhanced  $\alpha_v\beta_3$ -integrins targeted ultrasound imaging of ovarian tumor. A) Gross presentation of ovarian tumor in a hen diagnosed by  $\alpha_v\beta_3$ -integrins targeted contrast enhanced ultrasound imaging shown in figure 1. The tumor metastasized to other abdominal and peritoneal organs. B) Micrograph showing mucinous sub-type of ovarian carcinoma of the tumor shown in (A). Routine staining (hematoxylin and eosin) showed the tumor consists of ciliated columnar type of malignant cells with mucin (M)-like secretions in the lumen of the tumor glands. TS=tumor stroma.

mass-like structures in the ovary were maintained further to confirm that these small masses will develop large ovarian mass later. Representative sonograms from pre-and post-targeted CE-US indicating ovarian tumor mass are shown in **Figure 17 (A-F)**, gray scale, yellow dotted circle shows tumor border). At 3<sup>rd</sup> scan (45 weeks from the initial scan), 5 of the remaining 14 hens (including those two hens with small mass continued to monitor further) showed tumor associated changes in ovarian morphology or developed large solid mass and pixel intensities and Doppler indices in CE-DUS scanning indicative of OVCA. These hens were predicted to have OVCA. These hens were euthanized, gross morphology recorded and compared with targeted CE-US imaging. Tissue samples were processed for further studies. Remaining 9 apparently normal hens without any tumor related changes (in CE-DUS indices as well as in ovarian morphology) were monitored for another 8 weeks and final scanning of these hens were performed just before euthanasia at 53 weeks. At final scan, 4 hens showed OVCA related changes during scanning and they were predicted to have OVCA. These 4 hens together with the remaining 5 hens with apparently normal ovaries were euthanized at the end of the 53 weeks. Thus, compared to pre-targeted scans,  $\alpha_v\beta_3$ -integrins targeted contrast agent enhanced visualization of solid masses in the ovaries of 15 hens (selected for serum anti-NMP antibodies) on gray scale. Compared with healthy hens, the RI and PI values decreased gradually in hens developed OVCA even before the tumors were detectable by targeted CE-US imaging. The post-contrast RI and PI values for all of these hens with detectable solid ovarian mass were <0.44 and <0.96, respectively, similar to those reported previously in Specific Aims 1 and 2.

In hens without serum anti-NMP antibodies at the start of the study (n=20), ovarian tumor related morphologic abnormalities were not detected during  $\alpha_v\beta_3$ -integrins-targeted CE-US imaging.

**Tumor staging and histological sub-types of tumors:** Tumor staging and gross morphological features were recorded at euthanasia following targeted CE-US imaging prediction of suspected ovarian

malignancy related changes or at the end of the 53-weeks study period. Specimen from normal ovaries or ovaries with solid mass were processed for histopathological, immunohistochemical (paraffin and frozen), proteomic and molecular biological studies. Histological sub-types of tumors determined by hematoxylin-eosin staining (**Figure 18A-B**) and predictions of  $\alpha_v\beta_3$ -integrins-targeted CE-US imaging were compared and analyzed. All hens predicted to have ovarian tumor associated abnormalities during the  $\alpha_v\beta_3$ -integrins-targeted imaging were found to have a solid tissue mass limited to a part of their ovaries (n=13) (**Figure 19**) and metastasized to other organs (n=2) (**Figures 17-18**) confirming the diagnosis of  $\alpha_v\beta_3$ -integrins-targeted CE-US imaging. However, in two hens those were predicted to be normal and had no solid ovarian mass at gross observations found to have microscopic malignant features in their ovaries (**Figure 20**) indicating the false

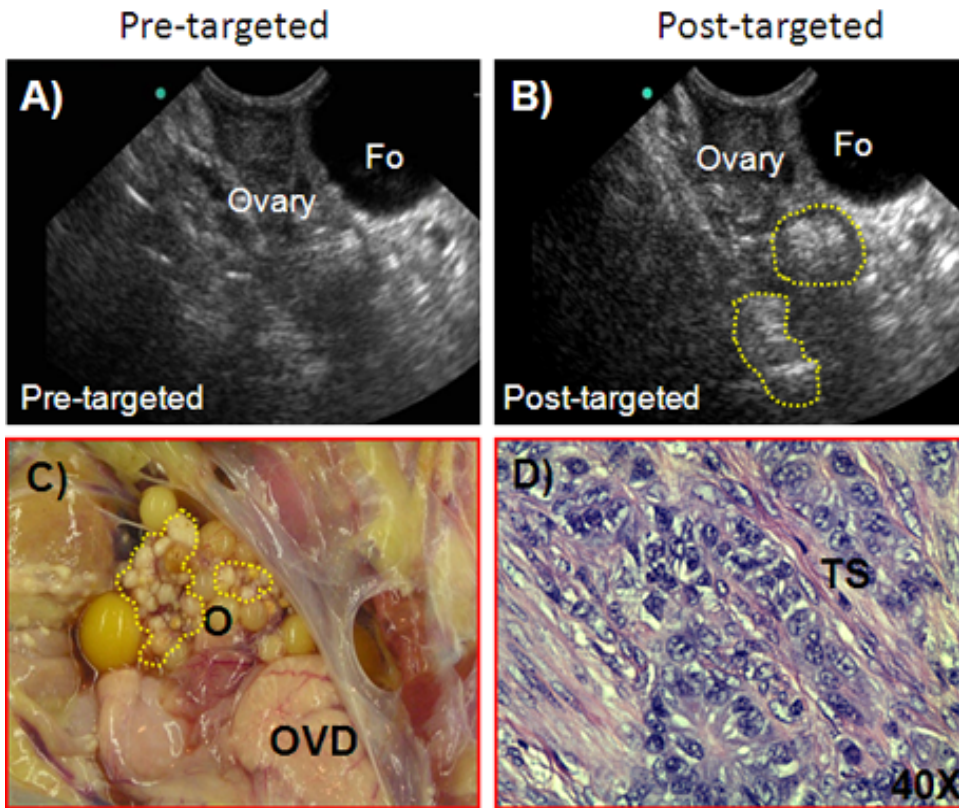


negative predictions and suggesting the failure of  $\alpha_v\beta_3$ -integrins-targeted imaging in detecting the microscopic ovarian carcinomas. Of the total 20 hens with serum anti-NMP antibodies, 17 developed ovarian tumors (including two microscopic) with serous (n= 8), endometrioid (n= 6) and mucinous (n=3) carcinomas. Thus, 17 of 20 hens (85%) with serum anti-NMP antibodies developed ovarian tumors and imaging indices for OVCA detection by  $\alpha_v\beta_3$ -integrins-targeted CE-US imaging established in specific Aim 1 detected 15 of 17 OVCA cases (88.24%) and failed to detect ovarian microscopic carcinomas in 2 of 17 hens that developed OVCA (with a false negative rate of 11.76%). It is assumed that these hens had underdeveloped ovarian TAN unable to be detected by  $\alpha_v\beta_3$ -integrins-targeted CE-DUS imaging. It is possible that had we monitor these hens further, these 3 normal hens with serum anti-NMP antibodies might have also developed OVCA eventually and would

have been detected by targeted CE-DUS imaging.

In hens without circulating anti-NMP antibodies group, as observed during targeted imaging, solid ovarian masses were not detected at gross examination at the end of the study period. However, histological examination revealed microscopic OVCA in 2 hens (1 of serous and 1 endometrioid carcinomas). Tumor related changes in targeted CE-US signal intensities or in their RI and PI values were not observed.

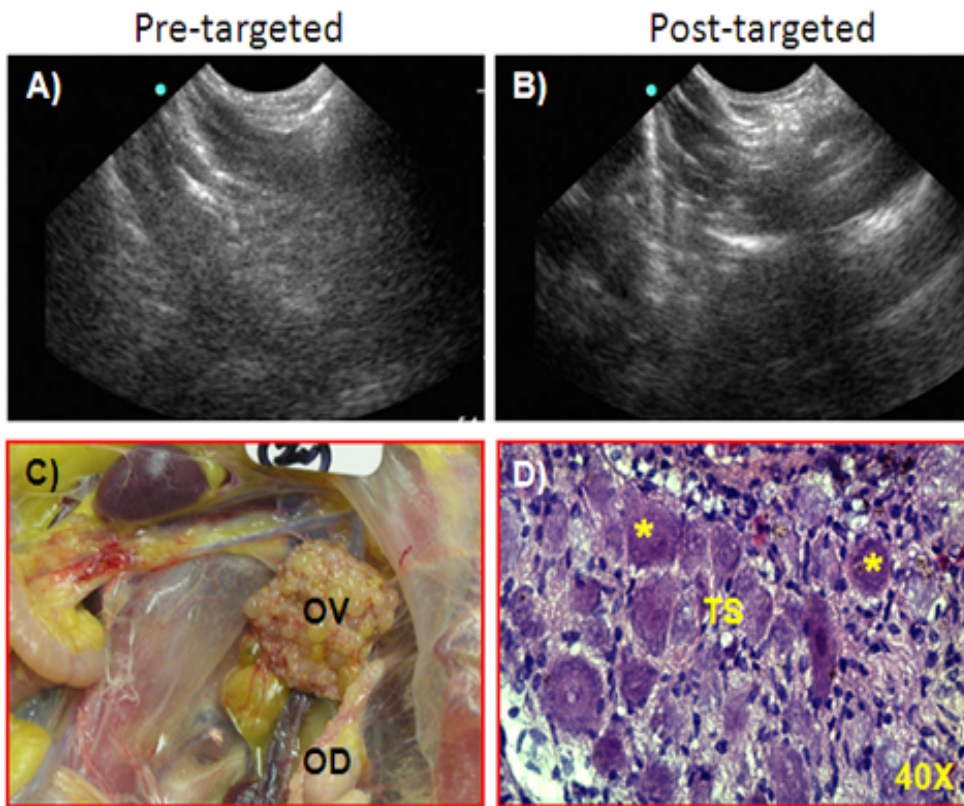
**Serum anti-NMP antibodies and OVCA development:** Presence of anti-NMP antibodies in serum of all hens in both groups collected at different scanning intervals were examined by immunoassay and confirmed by two-dimensional Western blotting (2D-WB) (**Figure 21**) as mentioned above [6]. Sera of young laying hens with normal egg laying rates were used as controls. Mean OD + 3SD of control hens were used as cut-off values as established in specific Aim 2. Hens with OD values greater than the cut-off value were considered as positive for circulating anti-NMP antibodies. All hens in anti-NMP positive group maintained their serum prevalence



**Figure 19:** Early detection of ovarian cancer in a hen by  $\alpha_v\beta_3$ -integrins contrast enhanced ultrasound imaging. The hen was monitored by  $\alpha_v\beta_3$ -integrins-targeted ultrasound scanning at 15-week interval for 45 weeks. A-B) Pre and post-targeted sonograms at 3<sup>rd</sup> scan (45 weeks from the start of the study). Pre-targeted sonogram showed apparently normal ovary with a preovulatory follicle (Fo). However, post-targeted imaging showed small solid mass-like (SM, yellow dotted line) structures in the ovary. C) Gross presentation of ovarian tumor diagnosed by  $\alpha_v\beta_3$ -integrins-targeted ultrasound imaging shown in (A). The tumor was limited in the ovary (O) with a little amount of ascitic fluid while the oviduct (OVD) was normal. D) Micrograph showing a serous histological sub-type of ovarian adenocarcinoma of the tumor shown in (A). Routine staining showed malignant cells containing large pleomorphic nuclei in a solid sheath of tumor stroma (TS) surrounded by fibromuscular layers. Thus,  $\alpha_v\beta_3$ -targeted imaging agents enhanced the ultrasound signal intensity for the detection of early stage ovarian tumors.

throughout the experimental period. However, in hens without circulating anti-NMP antibodies group, 4 hens developed anti-NMP antibodies over the course of the study period, 1 after 30-weeks (2<sup>nd</sup> scan) and 1 at 45-weeks (3<sup>rd</sup> scan) and 2 at 53 weeks (final scan). Immuno-proteomic analysis (2D-WB) using sera of these hens showed

specific immunoreactivity against tumor NMPs. Of these 4 hens, 2 had microscopic OVCA as mentioned above.

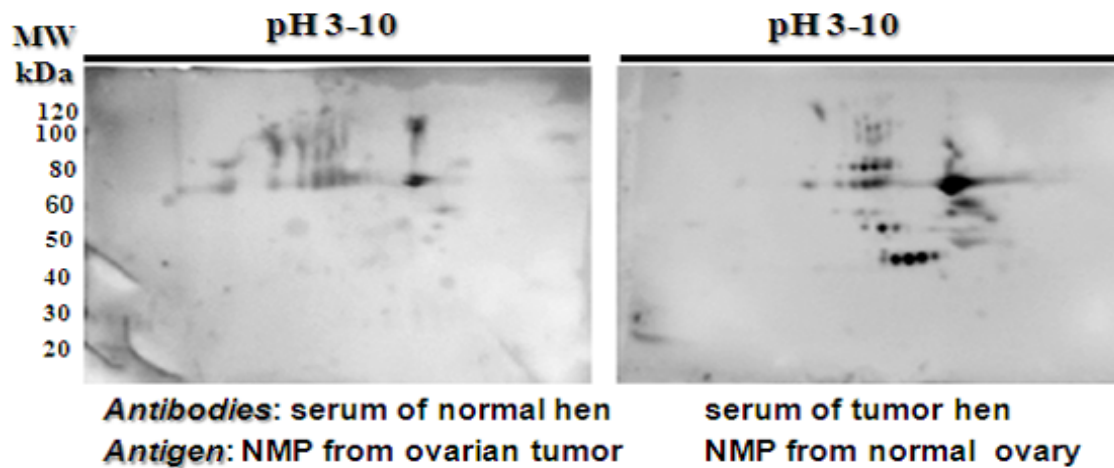


**Figure 20:** Limitations of  $\alpha_v\beta_3$ -integrins-targeted contrast enhanced ultrasound imaging agents in detecting microscopic ovarian cancer in a hen. This hen was monitored by  $\alpha_v\beta_3$ -integrins-targeted ultrasound scanning for 52 weeks. A-B) Pre- and post-targeted sonograms of hen ovary at 52 weeks (from the start of the study). The ovary appeared atrophied or regressed. Neither a preovulatory follicle nor a solid mass was detected in the ovary during both the pre- and post-targeted imaging. Moreover, remarkable differences were not observed in gray scale ovarian morphology between the pre- and post-targeted ultrasound imaging. C) Gross presentation showed atrophied ovary (OV) and oviduct (OD) without any detectable solid mass supporting the prediction of targeted ultrasound imaging. However, histological examination showed malignant focal lesions (\*) in the ovary suggesting the presence of microscopic ovarian carcinoma. Thus,  $\alpha_v\beta_3$ -integrins-targeted contrast enhanced ultrasound imaging agent could not detect microscopic carcinoma in the ovary. TS=tumor stroma.

**Immunohistochemical detection of  $\alpha_v\beta_3$ -integrins and DR6 (markers of ovarian tumor associated neo-angiogenesis, TAN):** Expression of  $\alpha_v\beta_3$ -integrins and DR6 by normal ovaries and ovaries with tumor was determined by immunohistochemistry as mentioned in specific Aims 1 and 2 and reported previously [9]. As described in specific Aims 1 and 2, similar patterns in the frequencies of  $\alpha_v\beta_3$ -integrins and DR6-expressing microvessels were significantly greater in hens with early stage OVCA than normal hens as shown in **Figure 16**. These results confirm that the greater signal intensities from hens with suspected ovarian tumor mass observed in the molecular targeted CE-US imaging were due to the presence of angiogenic microvessels in these hens. Although the frequencies of  $\alpha_v\beta_3$ -integrins and DR-6 expressing microvessels were higher in hens with microscopic OVCA than normal hens, however, the differences were not significant. These results suggest that ovarian TAN might not have fully established in these hens and that might be a reason for the inability of  $\alpha_v\beta_3$ -integrins-targeted CE-DUS imaging to detect these microscopic carcinoma.

Overall, taken together, specific Aim 3 showed that (1)  $\alpha_c\beta_H$ -targeted imaging indices established in specific Aim 1 detected ovarian tumors at early stage in laying hens in association with serum anti-NMP antibodies; (2) Increased signal intensity in  $\alpha_c\beta_H$ -targeted imaging intensities was associated with increased frequencies of  $\alpha_c\beta_H$ -integrins expressing microvessels; (3) Increase in the frequencies of DR-6 expression, a marker of ovarian TAN vessels was positively correlated with the prevalence of serum anti-NMP antibodies in hens with solid tumor mass but not in hens with microscopic OVCA. (4)  $\alpha_c\beta_H$ -targeted imaging indices could not detect hens with microscopic OVCA. Since targeted imaging detects microvessels expressing  $\alpha_c\beta_H$ -integrins and low frequency of  $\alpha_c\beta_H$ -expressing microvessels as seen by immunohistochemistry or underdeveloped tumor

associated angiogenesis in these hens may be one of the reasons of failure to detect microscopic carcinomas in the ovary.



**Figure 21.** Immunoreactivity of serum from normal or OVCA hens against nuclear matrix proteins (NMPs) from normal ovary or ovarian tumor detected by 2dimensional Western blotting (2D-WB). Compared with serum from normal hens (left panel), serum from OVCA hens showed strong reactivities (right panel) against ovarian NMPs indicating the prevalence of anti-ovarian NMP antibodies in OVCA hens. The size of the immunoreactive ovarian antigens ranged from 30-80kD.

### Key research accomplishment during the reporting period Year-3:

- ❖ The detectability of early stage OVCA by  $\alpha_v\beta_3$ -targeted imaging indices established in specific Aim 1 was tested.  $\alpha_v\beta_3$ -integrins-targeted CE-DUS indices established in specific Aim 1 detected all hens with early OVCA (tumor mass limited to a part of the ovary) but not ovarian microscopic carcinomas.
- ❖ Association of serum prevalence of anti-NMP antibodies and incidence and development of OVCA was tested. 85% hens with serum anti-NMP antibodies developed OVCA within 30-53 weeks suggesting its suitability as a surrogate marker of early OVCA detection.
- ❖ Association of DR-6 as a marker of ovarian tumor associated neoangiogenesis was examined. Compared with normal ovaries, expression of DR-6 increased in hens with early and late stages of OVCA. However, significant differences in DR-6 expression between normal and microscopic OVCA was not observed.

### REPORTABLE OUTCOMES (Year-3):

#### Manuscript in Press:

4. Á Barua A, Yellapa A, Bahr JM, Machado SA, Bitterman P, Basu S, Sharma S, Rotmensch J, and Abramowicz JS (2013). Enhancement of detection of ovarian tumors by  $\alpha_v\beta_3$ -integrins targeted ultrasound

molecular imaging agent in laying hens, a preclinical model of spontaneous ovarian cancer. *International Journal of Gynecological Cancer, In Press (appended at pages 31-67).*

**Presentation: Abstract published and presented:**

1. **Barua A**, Abramowicz JS, Bahr JM, Grasso SA, Sharma S, Basu S, Rotmensch J, Bitterman P (2013). Anti-NMP autoantibodies together with serum levels of death receptor 6 (DR6) detect ovarian tumors at early stage. Annual meeting of American Association for cancer Research (AACR), 2013, Washington D.C., April 5<sup>th</sup> –April 10<sup>th</sup>. Abstract # 3471 (appended at page 100)

**OVERALL RESEARCH ACCOMPLISHMENTS FROM SPECIFIC AIM1 TO AIM 3 PROJECT PERIOD:**

- ❖ **Established** the feasibility of the laying hen as a model of *in vivo* molecular targeted imaging for the detection of early ovarian tumor development ([10], appended at pages 31-67).
- ❖ **Established**, for the first time, the ability of  $\alpha_5\beta_1$ -integrins-targeted imaging agents to enhance the signal intensities of ovarian tumors in a spontaneous ovarian tumor ([10], appended at pages 31-67).
- ❖ **Confirmed** that  $\alpha_5\beta_1$ -integrins-targeted CE-US imaging agents predict ovarian tumor associated neoangiogenesis at early stage of spontaneous OVCA ([10], appended at pages 31-67)).
- ❖ **Serum anti-NMP antibodies** are an indicator of ovarian malignant nuclear transformation; ovarian tumor associated anti-NMP antibodies appear in serum before the tumor becomes detectable by targeted ultrasound imaging (appended at page 100);
- ❖ **This study** showed association of DR-6 with early stage OVCA ([11], appended at pages 68-76). This study further revealed DR-6 as a potential marker of tumor associated neoangiogenesis to be detected from serum and by detecting DR-6-expressing microvessles by DR-6-targeted ultrasound imaging. DR-6 can also be used as a novel target for anti-angiogenic drugs to inhibit ovarian tumor associated neoangiogenesis.
- ❖ **This study** also showed, for the first time, that tissue expression and serum levels of interleukin (IL)-16, a proinflammatory cytokine and a proangiogenic factor increased significantly in association with tumor associated neoangiogenesis and OVCA progression. Thus IL-16 can be an imaging target for the detection of malignant cells and ovarian TAN vessels associated with early stage OVCA ([12] appended at pages 77-85).
- ❖ **This study** also showed expression of immunoglobulin-like transcript (ILT)-3, a tumor induced immunosuppressant by malignant epithelium in association with OVCA development and progression ([13], appended at pages 86-92). Thus ILT3 may be used as a marker of targeted ultrasound imaging for the detection of malignant epithelium in association with  $\alpha_5\beta_1$ -integrins-targeted imaging agents. In addition, ILT3 can also be used to monitor the effects of chemotherapeutics and or OVCA recurrence.



- ❖ Finally,  $\alpha_v\beta_3$ -integrins-targeted CE-DUS imaging indices diagnostic of OVCA established in this study will be a foundation for a clinical study to test the efficacy of this imaging agent in detecting early stage OVCA in clinic.

## OVERALL REPORTABLE OUTCOMES DURING THE PROJECT LIFE (Year 1 to Year 3):

### Manuscripts: *In press* or Published

1. Barua A, Yellapa A, Bahr JM, Machado SA, Bitterman P, Basu S, Sharma S, Rotmensch J, and Abramowicz JS (2013). Enhancement of detection of ovarian tumors by  $\alpha_v\beta_3$ -integrins targeted ultrasound molecular imaging agent in laying hens, a preclinical model of spontaneous ovarian cancer. *International Journal of Gynecological Cancer*, *In Press* (**Appended at pages 31-67**).
2. Barua A, Yellapa A, Bahr JM, Abramowicz JS, Edassery SL, Basu S, Rotmensch J and Bitterman P (2012). Expression of death receptor 6 (DR6) by ovarian tumors in laying hens, a preclinical model of spontaneous ovarian cancer, *Translational Oncology*, *Translational Oncology*, 2012; 5 (4), 260-268. PMID: 22937178  
<http://www.transonc.com/future.php?vol=5&no=4> (**Appended at pages 68-76**)
3. Yellapa Y, Bahr JM, Bitterman P, Abramowicz JS, Edassery SL, Penumatsa K, Basu S, Rotmensch J and Barua A (2012). Association of Interleukin 16 with the development of ovarian tumor and tumor associated neo-angiogenesis in laying hen model of spontaneous ovarian cancer *International Journal of Gynecological Cancer*, 22(2):199-207. PMID: [22274315](https://pubmed.ncbi.nlm.nih.gov/22274315/) (**Appended at pages 77-85**)
4. Khan M, Bahr JM, Yellapa Y, Bitterman P, Abramowicz JS, Edassery SL, Basu S, Rotmensch J and Barua A (2012): Association of Immunoglobulin like transcript 3 (ILT3) with the progression of ovarian tumors in laying hens, a preclinical model of spontaneous ovarian cancer. *Translational Oncology*, 2012 April; 5(2): 85–91. PMID: [22496924](https://pubmed.ncbi.nlm.nih.gov/22496924/) (**Appended at pages 86-92**)

### Meeting presentations: Abstracts published and presented:

5. A. Barua, A. Yellapa, P. Bitterman, J. M. Bahr, S. Sharma, D. B. Hales, and J. L. Luborsky, J. S. Abramowicz (2011): Use of contrast-enhanced ultrasound imaging with microbubbles targeted to  $\alpha_v\beta_3$  integrins to enhance detection of early-stage ovarian tumors, ASCO Annual Meeting. Chicago, IL, USA; June 3-7, 2011. (**Appended in pages 93-94**)  
[http://www.asco.org/ascov2/Meetings/Abstracts?&vmview=abst\\_detail\\_view&confID=102&abstractID=84636](http://www.asco.org/ascov2/Meetings/Abstracts?&vmview=abst_detail_view&confID=102&abstractID=84636)
6. A. Yellapa, JS. Abramowicz, P. Bitterman, JM. Bahr, MJ. Bradaric, SL. Edassery, S. Sharma, A. Barua. Interleukin (IL-16) and tumor associated neo-angiogenesis detects ovarian cancer at early stage. AACR 102th Annual Meeting, Orlando, FL, April 2-6, 2011. (**Appended in pages 95**).  
<http://www.abstractsonline.com/Plan/ViewAbstract.aspx?sKey=0732f136-3e77-422d-886b-29769face7da&cKey=fc49e373-cefe-430d-ae3f-058a41a93bf4&mKey=%7b507D311A-B6EC-436A-BD67-6D14ED39622C%7d>

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[http://cancerres.aacrjournals.org/cgi/content/meeting\\_abstract/72/8\\_MeetingAbstracts/3646](http://cancerres.aacrjournals.org/cgi/content/meeting_abstract/72/8_MeetingAbstracts/3646)
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[http://cancerres.aacrjournals.org/cgi/content/meeting\\_abstract/72/8\\_MeetingAbstracts/5415](http://cancerres.aacrjournals.org/cgi/content/meeting_abstract/72/8_MeetingAbstracts/5415)
9. **Barua A.** Molecular targeted ultrasound imaging of  $\alpha_v\beta_3$ -integrins expressing microvessels in association with anti-NMP antibodies detects ovarian cancer at early stage. **Oral presentation-symposium on Ovarian Cancer: Prevention, Detection, & Treatment of the Disease and Its Recurrence: Molecular mechanism and Personalized Medicine, held from May 10-11, 2012, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. (Appended at pages 98-99)** <http://www.upci.upmc.edu/ovarian/index.cfm>
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#### PROBLEMS/CAVEATS OBSERVED DURING THE STUDY PERIOD:

This study detected all hens with early stage OVCA and ovarian tumor associated neoangiogenesis in which markers of ovarian TAN including  $\alpha_c\beta_H$ -integrins and DR-6 expressed by tumor associated microvessels. Tumors in all these hens were at early stage and limited to a part of the ovary. However, this study failed to detect ovarian microscopic carcinoma as tumor associated neoangiogenesis in these microscopic carcinomas were not well established to be detected by  $\alpha_c\beta_H$ -integrins targeted CE-DUS imaging. Therefore, detection of ovarian microscopic carcinomas may be improved by using  $\alpha_c\beta_H$ -integrins-targeted contrast agent in association with a serum marker of malignant ovarian transformation secreted by the malignant cells including anti-NMP antibodies.

#### TRANSLATIONAL SIGNIFICANCE:

The lack of an effective early detection test for ovarian cancer is one of the leading causes of highest rates of death of women with OVCA. Ultrasound imaging represents a potential non-invasive in vivo imaging modality for the detection of OVCA if its detection limit can be enhanced. Difficulty of identifying OVCA patients at early stage is one of the significant barriers to study and develop an early detection test. This study showed that the laying hen is a feasible model of studying the enhancement of OVCA detection by ultrasound imaging using targeted molecular imaging agents. No deleterious or toxic effects leading to abnormal physiology during or after the injection of  $\alpha_v\beta_3$ -integrins-targeted imaging agents were observed. Because the laying hen shares similar reproductive physiology, similar features of spontaneous OVCA and express similar molecular markers, results of this study



may directly be translated to the clinical study. Moreover, this study used ultrasound imaging system similar to those used in clinics. Thus, results of this study can be easily translated to clinic with least modification due to similar mechanical set up.

## OVERALL CONCLUSIONS:

The results of this study showed that contrast enhanced molecular imaging targeting ovarian  $\alpha_v\beta_3$ -integrins-expressing microvessels detected ovarian tumors at early stage when the tumor was limited to the ovary. This early detection of OVCA was based on the detection of  $\alpha_v\beta_3$ -integrins expressing ovarian tumor associated microvessels. However, microscopic ovarian malignant tumor lesions lacking with fully established ovarian tumor associated neo-angiogenesis cannot be detected by  $\alpha_v\beta_3$ -integrins-targeted ultrasound imaging. Changes in early stage ovarian cancer related CE-US imaging indices were associated with the elevation of serum levels of death receptor (DR6). Serum prevalence of anti-NMP antibodies was associated with tumor development and progression. Anti-NMP antibodies appeared in serum before the tumor become detectable through imaging. Thus serum anti-NMP antibodies may be used as a surrogate marker of ovarian malignant nuclear transformation together with  $\alpha_v\beta_3$ -integrins targeted CE-DUS imaging. This study also established the feasibility of laying hens as a model of non-invasive ultrasound imaging for testing the efficacy of novel imaging agents as well as targeted delivery of anti-OVCA drugs.

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## View Letter

Close

**Date:** 10/17/2013  
**To:** "Animesh Barua" animesh\_barua@rush.edu  
**From:** "" Beller.IJGC@ gmail.com  
**Subject:** IGC Decision

---

10/17/2013

RE: IGC-D-13-00021R1, entitled "Enhancement of ovarian tumor detection by <alpha><nu><beta>?-integrin-targeted ultrasound molecular imaging agent in laying hens, a preclinical model of spontaneous ovarian cancer"

Dear Dr. Barua,

I am pleased to inform you that your work has now been accepted for publication in International Journal of Gynecological Cancer. All manuscript materials will be forwarded immediately to the production staff for placement in an upcoming issue.

#### OPEN ACCESS

If you indicated in the revision stage that you would like your submission, if accepted, to be made open access, please go directly to step 2. If you have not yet indicated that you would like your accepted article to be open access, please follow the steps below to complete the process:

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  - b. Manuscript Number - IGC-D-13-00021R1

Thank you for submitting your interesting and important work to the journal.

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Your username is: \*\*\*\*\*

Your password is: \*\*\*\*\*

With Kind Regards,

Professor Uzi Beller  
 Editor-in-Chief  
 International Journal of Gynecological Cancer

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# International Journal of Gynecological Cancer

## Enhancement of ovarian tumor detection by $\alpha\beta_3$ -integrin-targeted ultrasound molecular imaging agent in laying hens, a preclinical model of spontaneous ovarian cancer

--Manuscript Draft--

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|------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Manuscript Number:</b>                            | IGC-D-13-00021R1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <b>Full Title:</b>                                   | Enhancement of ovarian tumor detection by $\alpha\beta_3$ -integrin-targeted ultrasound molecular imaging agent in laying hens, a preclinical model of spontaneous ovarian cancer                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| <b>Short Title:</b>                                  | $\alpha\beta_3$ -integrin targeted imaging of ovarian tumors                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| <b>Article Type:</b>                                 | Original Study                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| <b>Keywords:</b>                                     | ovarian cancer; early detection; molecular imaging, $\alpha\beta_3$ -integrins; animal model                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| <b>Corresponding Author:</b>                         | Animesh Barua, PhD<br>Rush University Medical Center<br>Chicago, Illinois UNITED STATES MINOR OUTLYING ISLANDS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
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| <b>Corresponding Author's Institution:</b>           | Rush University Medical Center                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| <b>Corresponding Author's Secondary Institution:</b> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| <b>First Author:</b>                                 | Animesh Barua, PhD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
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|                                                      | Aparna Yellapa, MS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|                                                      | Janice M Bahr, PhD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
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|                                                      | Sanjib Basu, PhD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|                                                      | Sameer Sharma, M.D.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
|                                                      | Jacques S Abramowicz, M.D.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
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| <b>Manuscript Region of Origin:</b>                  | USA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| <b>Abstract:</b>                                     | <p><b>Objective:</b> Due to the lack of an effective early detection test, ovarian cancer (OVCA) in most cases is detected at late stages and remains a fatal gynecological malignancy. Molecular imaging provides information on the changes associated with the development of a disease at molecular levels. Because angiogenesis is an early event in tumor development, increased expression of <math>\alpha\beta_3</math>-integrins by ovarian tumor-associated angiogenic (TAN) microvessels provides a target for non-invasive ultrasound imaging to detect early stage OVCA. The goal of this study was to examine the feasibility of <math>\alpha\beta_3</math>-integrin-targeted molecular imaging agent in enhancing the detection of spontaneous ovarian tumor in laying hen, a preclinical model of OVCA.</p> <p><b>Methods:</b> The study was conducted in two phases including a cross sectional exploratory one followed by a prospective monitoring of hens for 45 weeks with targeted ultrasound imaging. Changes in ultrasound signal intensity were determined before and after injection of <math>\alpha\beta_3</math>-integrin-targeted imaging agent in hens with spontaneous OVCA. All images were digitally stored. Following scanning, ovarian tissues from all hens were collected and processed for histopathological and immunohistochemical studies.</p> |

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|-------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                               | <p>Results: Ultrasound signal intensity was significantly (<math>P &lt; 0.001</math>) higher in hens with early stage OVCA than normal hens and increased further in late stage OVCA. Compared with normal, ultrasound signal intensities increased approximately 19 fold in early stage and 26 fold in late stage OVCA. Differences in signal enhancement were not observed among different histological sub-types of OVCA. Higher signal intensities from targeted imaging of ovarian tumors were associated with increased number of <math>\alpha v\beta_3</math>-integrin expressing ovarian microvessels. Prospective monitoring of hens with <math>\alpha v\beta_3</math>-integrin-targeted imaging agent detected OVCA at early stage. Conclusions: These results suggest that <math>\alpha v\beta_3</math>-integrin-targeted imaging agent enhanced the visualization of ovarian TAN microvessels in hens with early stage OVCA and may form a foundation for clinical studies.</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| <b>Response to Reviewers:</b> | <p>Responses to Reviewers comments and suggestions:<br/> Reviewer # 1: We are very thankful and appreciate your time to review our manuscript. We have revised the manuscript according to your comments and suggestions. Changes in response to specific comments are mentioned below:</p> <p>Comment 1. In the prospective monitoring study, the methods say that hens appearing normal were also euthanized at the study endpoint (45 weeks). However, no description of gross exam at necropsy is included in the results for these animals. This data is essential to understanding the performance of your imaging approach. Did any of the hens that appeared normal by imaging have tumors identified during necropsy?</p> <p>Response: Gross morphology of hens predicted to be normal in TVUS scanning is included in Lines (L) 325-326 of the revised version.</p> <p>Comment 2. The discussion mentions that you "did not use animals with benign ovarian tumors". However, your methods do not describe excluding animals with benign tumors. Were additional animals excluded from the analysis? This may be appropriate, but should be described in detail in the methods section.</p> <p>Response: No animal was excluded from the study. The reason of not using animals with benign tumors is explained in L388-389 of the revised version.</p> <p>Comment 3. It would be helpful to know how much variability is present or expected for this targeted imaging agent. For example, if you identified hens with suspected ovarian tumors and then re-injected/re-imaged on 3 consecutive days, would you see consistent indications of a tumor?</p> <p>Response: In the prospective study once an animal was suspected for ovarian tumor, it was euthanized immediately. Thus, an animal with ovarian cancer was not scanned more than one time to determine the variability of the targeted imaging agent.</p> <p>Comment 4. Please provide additional information on RI and PI in the methods section for readers who are not experts in ultrasound imaging. RI and PI values are described in the text of the results section, but only RI is shown in Figure 5.</p> <p>Response: Additional information on RI and PI value is included in L167-170. Only RI values are mentioned in the Figure 5. Generally changes in RI values also indicate what would be the possible PI values. Thus using RI values only is also an accepted method for describing tumor-related changes in blood flow characteristics.</p> <p>Comment 5. In order to be up to date, the authors should consider including the most recently published ovarian screening study on CA-125 and TVUS imaging to the section on this topic in the introduction (just published this month).</p> <p>Response: An updated reference on CA-125 and TVUS is included in L84 of the revised version.</p> <p>Once again, we appreciate your time for reviewing our manuscript and thank you very much.</p> <p>Reviewer #2: The introduction is wordy and too long and should be shortened.<br/> Response: We are very grateful for your suggestions and comments on our manuscript. We have revised and shortened the introduction in the revised version. Once again thank you very much for your helps.</p> |

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## RUSH UNIVERSITY MEDICAL CENTER

September 25, 2013

Uziel Beller, MD,  
Editor-in-Chief,  
*International Journal of Gynecological Cancer*  
Editorial Office

Dear Dr. Beller,

We appreciate your consideration to review our manuscript for publication. I am submitting the revised version of the manuscript entitled "*Enhancement of detection of ovarian tumors by  $\alpha_v\beta_3$ -integrins-targeted ultrasound molecular imaging agent in laying hens, a preclinical model of spontaneous ovarian cancer*" (IGC-D-13-00021).

We have revised the manuscript according to the comments and suggestions of the reviewers. All changes are marked red in the revised version.

Thank you very much for your helps.

Sincerely,

A handwritten signature in black ink, appearing to be 'Animesh Barua', written over a horizontal line.

Animesh Barua, Ph.D.

# **Enhancement of ovarian tumor detection by $\alpha_v\beta_3$ -integrin-targeted ultrasound molecular imaging agent in laying hens, a preclinical model of spontaneous ovarian cancer**

Animesh Barua, PhD<sup>\* § ‡</sup>, Aparna Yellapa, MS<sup>\*</sup>, Janice M Bahr, PhD<sup>†</sup>, Sergio A Machado, DVM<sup>†</sup>, Pincas Bitterman, MD<sup>‡ §</sup>, Sanjib Basu, PhD<sup>||</sup>, Sameer Sharma, MD<sup>\* §</sup> and Jacques S Abramowicz, MD<sup>§, Á</sup>

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**Short title:  $\alpha_v\beta_3$ -integrin targeted imaging of ovarian tumors**

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## **Support**

This study was supported by the United States Department of Defense Ovarian Cancer Research Program under award number (W81XWH-10-1-0523).

**Enhancement of ovarian tumor detection by  $\alpha_v\beta_3$ -integrin-targeted  
ultrasound molecular imaging agent in laying hens, a preclinical model of  
spontaneous ovarian cancer**

Animesh Barua, PhD<sup>\*§‡</sup>, Aparna Yellapa, MS<sup>\*</sup>, Janice M Bahr, PhD<sup>†</sup>, Sergio A Machado,  
DVM<sup>†</sup>, Pincas Bitterman, MD<sup>‡§</sup>, Sanjib Basu, PhD<sup>||</sup>, Sameer Sharma, MD<sup>\*§</sup> and Jacques S  
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**Support**

This study was supported by the United States Department of Defense Ovarian Cancer Research  
Program under award number (W81XWH-10-1-0523).



**Objective:** Due to the lack of an effective early detection test, ovarian cancer (OVCA) in most cases is detected at late stages and remains a fatal gynecological malignancy. Molecular imaging provides information on the changes associated with the development of a disease at molecular levels. Because angiogenesis is an early event in tumor development, increased expression of  $\alpha_v\beta_3$ -integrins by ovarian tumor-associated angiogenic (TAN) microvessels provides a target for non-invasive ultrasound imaging to detect early stage OVCA. The goal of this study was to examine the feasibility of  $\alpha_v\beta_3$ -integrin-targeted molecular imaging agent in enhancing the detection of spontaneous ovarian tumor in laying hen, a preclinical model of OVCA.

**Methods:** The study was conducted in two phases including a cross sectional exploratory one followed by a prospective monitoring of hens for 45 weeks with targeted ultrasound imaging. Changes in ultrasound signal intensity were determined before and after injection of  $\alpha_v\beta_3$ -integrin-targeted imaging agent in hens with spontaneous OVCA. All images were digitally stored. Following scanning, ovarian tissues from all hens were collected and processed for histopathological and immunohistochemical studies.

**Results:** Ultrasound signal intensity was significantly ( $P < 0.001$ ) higher in hens with early stage OVCA than normal hens and increased further in late stage OVCA. Compared with normal, ultrasound signal intensities increased approximately 19 fold in early stage and 26 fold in late stage OVCA. Differences in signal enhancement were not observed among different histological sub-types of OVCA. Higher signal intensities from targeted imaging of ovarian tumors were associated with increased number of  $\alpha_v\beta_3$ -integrin expressing ovarian microvessels. Prospective monitoring of hens with  $\alpha_v\beta_3$ -integrin-targeted imaging agent detected OVCA at early stage.

**Conclusions:** These results suggest that  $\alpha_v\beta_3$ -integrin-targeted imaging agent enhanced the

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56 visualization of ovarian TAN microvessels in hens with early stage OVCA and may form a  
57 foundation for clinical studies.

58 **Key words:** Ovarian cancer; early detection; molecular imaging,  $\alpha_v\beta_3$ -integrins; animal model

Although the survival rates of patients with several malignancies have increased in recent years, ovarian cancer (OVCA) remains a fatal disease of women with the highest rate of mortality among gynecological malignancies<sup>1</sup>. Because of non-specificity of symptoms at early stages and the lack of an effective early detection test, OVCA in most cases is diagnosed at late stages with a 5-year survival rate of <30% as opposed to 80-90% when detected at early stages<sup>2, 3</sup>. Serum levels of CA-125 together with traditional transvaginal ultrasound (TVUS) scanning, although proved ineffective, are currently available and used methods for the detection of OVCA[4]. CA-125 is non-specific to OVCA in its early stage and 20% of patients do not show elevated levels of CA-125 at all<sup>4</sup>. Due to the limited resolution, using traditional TVUS scanning together with CA-125 did not improve the overall detection rates of OVCA as no imaging targets in the ovary corresponding to the elevated serum CA-125 levels have been defined<sup>5, 4</sup>. Thus identification of a tumor associated target(s) diagnostic of early stage OVCA as well as enhancement of TVUS resolution to detect those target(s) is essential to establish an early detection test for OVCA.

Tumor-associated neo-angiogenesis (TAN) is one of the earlier events in tumorigenesis during which the tumor induces the development of immature microvessels from the existing vessels for its supply of nutrition through blood<sup>6-8</sup>. TAN microvessels overexpress  $\alpha_v\beta_3$ -integrins on their surface offering a potential target for the detection of OVCA at an early stage<sup>9</sup>.  $\alpha_v\beta_3$ -integrin expressing TAN microvessels can be detected *in vivo* if a targeted imaging agent can be developed and the current detection limit of TVUS can be enhanced. Contrast-enhanced ultrasonography using ultrasound contrast agents has improved the visualization and quantification of tumor vascularity<sup>10-15</sup>. Substantial studies on the development of ultrasound microbubble contrast agents to target tumor vasculature specifically, by conjugating peptides or antibodies have been performed<sup>16, 17</sup>.  $\alpha_v\beta_3$ -integrin- targeted microbubbles are one of such

targeted imaging probes developed for the imaging of angiogenic microvessels in cardiomyopathy, atherosclerosis and peritoneal arterial diseases<sup>18, 19</sup>. Application of these targeted microbubbles have been tested using tumor cell lines and animal models of human xenograft solid tumors by different imaging modalities including positron emission tomography (PET), magnetic resonance imaging (MRI) and ultrasound<sup>9, 20-24</sup>. However, induced subcutaneous tumors differ remarkably with spontaneous ovarian tumors in their anatomical location and tumor vasculature. Furthermore, the dynamics of imaging agents and the pattern of ultrasound signal from a deeper anatomical location of an organ like the ovaries also differ greatly from those of subcutaneous tumor mass. Thus, information on the suitability of  $\alpha_v\beta_3$ -integrin-targeted microbubbles for the detection of *spontaneous* OVCA by ultrasound imaging in a preclinical model may be useful for clinical studies. However, the lack of a preclinical *in vivo* animal model of *spontaneous* OVCA is a significant barrier to test the suitability of  $\alpha_v\beta_3$ -integrin-targeted microbubble imaging probes.

We and a few other groups have reported high rates of spontaneous OVCA incidence in laying hens<sup>25, 26</sup> with a significant proportion of hen OVCA originating from the oviduct<sup>25, 27, 28</sup>. Similar to OVCA in humans, epithelial ovarian tumors in hens are of four histological sub-types (serous, endometrioid, mucinous and occasionally clear cell carcinoma)<sup>29</sup>. The incidence of serous and endometrioid OVCA are higher than those of mucinous and clear cell OVCA. Ovarian tumors in hens also express several markers similarly expressed by human OVCA<sup>30-34</sup>. We adapted TVUS imaging method for the detection of ovarian tumors in hens<sup>25</sup>. Thus the laying hen is a highly innovative model to test the feasibility of  $\alpha_v\beta_3$ -integrin-targeted imaging probes for the detection of spontaneous OVCA at an early stage by non-invasive *in vivo* TVUS imaging. Therefore, the goals of this study were to: 1) examine whether  $\alpha_v\beta_3$ -integrin-targeted

imaging probes enhance the visualization of spontaneous ovarian tumors; and 2) to test whether these imaging probes can detect ovarian tumors at an early stage in laying hens, a preclinical model of spontaneous OVCA. Enhancement of visualization of spontaneous ovarian tumors was examined in an exploratory study and the feasibility of early detection of ovarian tumors was examined in a prospective study.

## MATERIALS AND METHODS

### Animals

A flock of commercial strains of White Leghorn laying hens (*Gallus domesticus*, 3 years old) were reared under standard poultry care and management and provided with feed and water *ad libitum*. The egg-laying rates (an indicator of ovarian function; a low egg-laying rate indicates decreased ovarian function) of the hens were recorded on a daily basis. The normal rate of egg laying by a commercial laying hen is more than 250 eggs per year, and less than 50% of the normal laying rate is considered a low egg-laying rate<sup>26</sup>. Hens (n = 90) with low or irregular egg-laying rates and those that stopped laying, with or without any abdominal distention (a sign of possible ovarian tumor-associated ascites), were selected randomly from the flock for  $\alpha_v\beta_3$ -integrin-targeted TVUS imaging. The incidence of ovarian cancer in laying hens of this age group was reported to be approximately 15% to 20% and is associated with low or complete cessation of egg laying<sup>26, 29, 31</sup>. All procedures were performed according to Institutional Animal Care and Use Committee approved protocol.

### $\alpha_v\beta_3$ -Integrin- Targeted Imaging Probes

$\alpha_v\beta_3$ -integrin- targeted TVUS imaging of hen ovaries was performed using Visistar® Integrin (Targeson, Inc San Diego, CA). Visistar Integrin is a microsphere ultrasound contrast agent



containing a cyclic RGD (Arginine-Glycine-Aspartic acid) targeting ligand<sup>9</sup>. This microsphere is known to bind to  $\alpha_v\beta_3$ -integrins found on angiogenic vascular endothelium.

## **Sonography**

### **Pre-targeted imaging**

Sonography was performed in a continuous pattern before, during and after the injection of targeted imaging probes as reported previously with little modification<sup>14, 26</sup>. Briefly, all hens were scanned using an instrument equipped with a 1 to 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA). Each hen was immobilized and gently restrained by an assistant. Transmission gel was applied to the surface of the transducer; a probe cover was applied; and gel was reapplied to the covered probe to ensure uninterrupted conductance of the sound waves. The transducer was inserted approximately at a 30° angle to the body, 3 to 5 cm into the vagina and 2-dimensional (2D) transvaginal gray scale and pulsed Doppler sonography were performed. Young egg-laying hens (because the ovaries of these hens contain more developing follicles compared to old hens) were used as standard controls for mechanical adjustment to reveal and characterize the fully functional normal ovaries of hens. The area of a tumor to be imaged was determined according to 3 conditions as reported previously<sup>14</sup>: (1) the whole tumor should be seen on the image, if possible; (2) the sectional plane should contain the solid part (wall, septa, and papillae) of the tumor; and (3) the most vascularized area was selected. For normal ovaries, ovaries without any detectable tumor, and atrophic ovaries, the region surrounding the ovary was scanned, and the transducer was swept through the entire area for complete scanning of the ovary. Gray scale morphologic evaluation of the ovarian mass was performed with attention to the number of preovulatory follicles, the presence of abnormal-looking follicles, septations,

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4 164 papillary projections or solid areas, and echogenicity. After morphologic evaluation, the color  
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6 165 Doppler mode was activated for identification of vascular color signals. Once a vessel was  
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9 166 identified on color Doppler imaging, pulsed Doppler was activated to obtain a flow velocity  
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12 167 waveform. Doppler indices (resistive index,  $RI = [\text{systolic velocity} - \text{diastolic velocity}] / \text{systolic}$   
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14 168  $\text{velocity}$  and pulsatility index,  $PI = [\text{systolic velocity} - \text{diastolic velocity}] / \text{mean}$ ) were  
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16 169 automatically calculated from at least 2 consecutive samples (2 separate images from the same  
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19 170 ovary) and the average RI and PI values were used for analysis. All images were processed and  
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## 25 172 **Targeted imaging**

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29 173 Targeted imaging was performed according to the manufacturer's instructions as reported  
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31 174 earlier<sup>9, 14, 26</sup>. A preliminary experiment was conducted with  $\alpha_v\beta_3$ -integrins targeted or isotype  
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34 175 control microbubbles using 10 animals containing fully functional ovaries to adjust the  
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36 176 mechanical index and determine the optimum dosage of microbubbles. To confirm that the signal  
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39 177 acquired after targeted microbubble injection was from microbubbles-bounded target tissue,  
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41 178 images following the delivery of a destructive pulse with high mechanical index were taken and  
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43 179 compared with non-destructive targeted images for signal intensities.  
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47 180 The dose of 10  $\mu\text{L/kg}$  body weight was found optimal for better resolution in the  
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50 181 preliminary experiment. Targeted microbubbles were prepared before injection according to the  
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52 182 manufacturer's directions. Briefly, the vial containing the microbubble suspension was inverted  
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54 183 and gently rotated to resuspend the microspheres completely. The suspension was transferred  
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57 184 from the vial by an injection syringe with a 19-gauge needle to a angiocatheter (small-vein  
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59 185 infusion set, female luer, 12-in tubing, 25-gauge needle; Kawasumi Laboratories, Tampa, FL)  
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4 186 containing 100  $\mu$ L of 0.9% sodium chloride previously inserted into the left wing vein (brachial  
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7 187 vein) of the hen and followed by the reloading of 1 mL of a 0.9% sodium chloride solution. The  
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9 188 loading of the sodium chloride solution before and after injection of microbubbles helped  
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11 189 maintain the vascular patency and airtight condition, in addition to flushing the bubbles from the  
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14 190 hen's circulation. The area imaged during pre-targeted imaging was imaged again after targeted  
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16 191 microbubble injection. All images were archived digitally in a still format as well as real-time  
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19 192 clips (15 minutes for each hen) on single-sided recordable digital video disks (DVD+R format;  
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21 193 Maxell Corporation of America, Fair Lawn, NJ) readable on a personal computer.  
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#### 25 194 **Evaluation of the Effect of Targeted Microbubbles**

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29 195 The effect of targeted microbubbles was evaluated visually during the examination and the  
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31 196 enhancement of microvessel detection by targeted imaging was assessed afterward from  
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33 197 reviewing the archived video clips. After review of the complete clips, the region of interest  
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36 198 (ROI) was selected by drawing on the ovarian stroma of normal hens or on the area containing  
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38 199 solid tissue mass in OVCA hens. In normal hens, areas containing large developing follicles  
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41 200 were avoided during the selection of ROIs. The signal intensity of the selected area was  
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43 201 measured in pixel values using a computer-assisted software program (MicroSuite version 5;  
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45 202 Olympus Corporation, Tokyo, Japan). Using the software, the intensity of the ROI (sum of the  
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48 203 pixel values within the region of interest) was measured from the pre-targeted and targeted  
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51 204 image. The net contrast enhancement ( $CE = C_t - C_{pt}$ ) was determined and the CE ratio (CER) was  
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53 205 calculated using the following equation:  $CER = (C_t - C_{pt}) / C_{pt} \times 100\%$  where  $C_{pt}$  = pixel intensities  
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55 206 from ROI of pre-targeted image and  $C_t$  = pixel intensities from ROI of targeted image.  
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## **Ovarian Morphologic Evaluation**

All hens were euthanized after targeted imaging and examined for the presence of a solid mass in the ovary and any other organs, ascitic fluid, preovulatory follicles, and atrophy of the ovary, as reported previously. Gross observation was compared with the sonographic evaluations and photographed. A normally functional ovary had viable preovulatory follicles (more detailed information on hen ovarian physiology has been published elsewhere<sup>26, 29</sup>, whereas no large follicles or visible lesions were found in normal hens that stopped egg laying. Tumor staging was performed according to the gross metastatic status, as reported previously<sup>29</sup>. Briefly, early OVCA was characterized by detectable formation of solid tumor limited to the ovary. Late stages of OVCA were characterized by tumor metastasis to distant organs with moderate to extensive ascites.

## **Ovarian Histopathologic Evaluation and Immunohistochemical Detection of Ovarian Microvessel Density**

Representative portions of a solid ovarian mass or the whole ovary (in cases of atrophic or grossly normal-appearing ovaries) were divided into several blocks, processed for paraffin or frozen sections, and stained with hematoxylin-eosin. Microscopic tumor (if present) in any part of the ovary was detected by routine histologic examination with hematoxylin-eosin staining, and tumor types were determined by light microscopy, as reported previously<sup>29</sup>.

After histopathologic examination, frozen sections (6  $\mu$ m thick) of normal and malignant ovaries of all stages and types were processed for routine immunohistochemistry to assess the tumor-associated microvessel density using a monoclonal anti- $\alpha_v\beta_3$ -integrins antibody (primary antibody; Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. The frequencies

of  $\alpha_v\beta_3$ -expressing microvessels were determined from the tumor vicinity or ovarian stroma of normal hens (excluding the follicular areas), as reported earlier<sup>35</sup> using a light microscope attached to digital imaging stereological software (MicroSuite version 5; Olympus Corporation) with little modification. Briefly, immunostained slides were examined at low-power magnification ( $\phi 10$  objective and  $\phi 10$  ocular) to identify the areas of maximum neovascularization of the tumor. Vessels with thick, regular, and complete muscular walls as well as vessels with large lumina were excluded from the count, as reported previously<sup>35</sup>. In each section, the 5 most vascular areas were chosen. The number of microvessels in a 20,000- $\mu\text{m}^2$  area was counted at an  $\phi 40$  objective and  $\phi 10$  ocular magnification. The averages of these sections were expressed as the number of immunopositive microvessels in a 20,000- $\mu\text{m}^2$  area of a normal or tumorous ovary. Tumor histologic and immunohistochemical observations were compared to the sonographic predictions.

#### **Prospective Monitoring of Hens by $\alpha_v\beta_3$ -Integrin- Targeted Ultrasound Imaging to Detect Ovarian Tumor Development in Hens**

Three years old 20 hens with low egg laying rates and 20 hens with normal laying rates without any detectable ovarian abnormality were selected by  $\alpha_v\beta_3$ -integrin-targeted ultrasound imaging mentioned above. Hens were monitored by  $\alpha_v\beta_3$ -integrin-targeted ultrasound scanning at 15 week intervals up to 45 weeks. Gray scale morphology as well as Doppler indices (RI and PI values) for each hen was recorded at each scan. Hens were scanned with reference to the tumor associated imaging parameters observed in the exploratory study mentioned above (including changes in ultrasound signal intensities and RI and PI values). Any hen suspected for ovarian tumor during scanning at any interval was sacrificed and those appeared normal during imaging



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4 251 were euthanized at the end of the monitoring period (45 weeks). Serum samples from all hens  
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6 252 were collected at each interval. Ultrasound prediction was confirmed and gross pathology was  
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9 253 noted at necropsy and ovarian specimens were collected and processed as mentioned earlier for  
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11 254 further use.  
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## 15 255 **Statistical Analysis**

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17 256 Descriptive statistics for imaging parameters were determined, and statistical analysis was  
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20 257 performed in SPSS version 15 (SPSS Inc, Chicago, IL) and the R statistical software  
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22 258 (<http://www.r-project.org>). The differences in the net ultrasound signal intensities and the  
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25 259 frequencies of  $\alpha_v\beta_3$ -integrins expressing microvessels among normal hens or hens with early and  
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27 260 late stage OVCA were analyzed by the two-samples *t* test and the exact Mann-Whitney test. The  
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30 261 association between the net ultrasound signal intensity and the frequency of  $\alpha_v\beta_3$ -integrins  
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32 262 expressing microvessels was examined by Pearsons coefficient of correlations. *P* < 0.05 was  
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35 263 considered significant. All reported *P* values are 2 sided.  
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## 39 264 **RESULTS**

### 40 41 42 265 **Non-invasive targeted ultrasound imaging**

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44 266 On pre-targeted and targeted imaging, multiple preovulatory follicles and small growing stromal  
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47 267 follicles were observed in normal hens with functional ovaries. Compared to pre-targeted  
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50 268 ovaries, visualization of solid ovarian masses with or without projected septa and papillary  
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52 269 structures, accompanying ascites, or both were enhanced remarkably in the ovaries of 15 hens  
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54 270 (Fig. 1). Of these 15 hens, 8 had solid masses in the ovary together with profuse ascites and were  
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57 271 predicted to have late-stage OVCA. In the remaining 7 hens, solid masses were limited to a part  
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60 272 of the ovary with no or little ascites, and they were provisionally categorized as early-stage  
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OVCA. Imaging predictions and the stage of the tumor were confirmed during gross examination of hens at necropsy (Fig. 2).

Overall, compared to pre-contrast, the pixel intensity of the signals from the ovaries with tumor increased remarkably after the injection of targeted microbubbles. The enhancement in signal intensity for normal hens ( $n = 25$ ) was  $1,651.89 \pm 563.79$  (mean  $\pm$  SD) pixel<sup>2</sup> in 1500- $\mu\text{m}^2$  area of ROI and it was significantly higher ( $P < 0.001$ ) in hens with early stage OVCA ( $22,277.20 \pm 807.15$  pixel<sup>2</sup> in 1500- $\mu\text{m}^2$  area of ROI) ( $n = 7$ ) and late stage OVCA ( $25,130.35 \pm 953.73$  pixel<sup>2</sup> in 1500- $\mu\text{m}^2$  area of ROI) ( $n = 8$ ). Similarly, compared with normal hens, the rates of enhancement of signal intensities due to targeted imaging were approximately 19 fold in hens with early stage OVCA and increased further (approximately 27 fold) in hens with late stage OVCA. Thus, compared to pre-contrast scans, targeted imaging by  $\alpha_v\beta_3$ -integrin-targeted contrast agent enhanced visualization of solid masses in the ovaries of hens on gray scale.

### Histopathologic Findings and Microvessel Density

Presence of tumors and their histological sub-types were confirmed by routine histologic examination with hematoxylin-eosin staining (Fig. 2). Among the 8 hens with late-stage OVCA, 4 had serous, 3 had endometrioid and 1 had mucinous carcinoma. Among the 7 hens at early stage OVCA, 3 were serous, 3 were endometrioid, and 1 was mucinous.

$\alpha_v\beta_3$ -integrins expressing microvessels were detected in both normal ovaries and ovaries with tumor (Fig. 3, *upper panel*). In normal ovaries, very few  $\alpha_v\beta_3$ -integrin expressing microvessels were seen in the follicular theca and the ovarian stroma. Compared with normal ovary, many  $\alpha_v\beta_3$ -integrin expressing microvessels were localized in the tumor vicinity (spaces

between tumor glands) in hens with OVCA. Occasionally ovarian tumor epithelium also expressed  $\alpha_v\beta_3$ -integrins. The population of  $\alpha_v\beta_3$ -integrin expressing microvessels in hens with early stage OVCA was significantly ( $P<0.001$ , exact Mann-Whitney test) higher (mean  $\pm$  SD=  $6.67 \pm 0.75$  in  $20,000\text{-}\mu\text{m}^2$  of tumor tissue) than that of normal hens ( $2.7 \pm 0.60$  in  $20,000\text{-}\mu\text{m}^2$  of ovarian stromal tissue), and increased further in hens with late stage OVCA ( $10 \pm 1.24$  in  $20,000\text{-}\mu\text{m}^2$  of tumor tissue) (Fig. 3, *bottom panel*). However, differences were not observed among different histological sub-types of ovarian tumors with regard to the frequencies of their  $\alpha_v\beta_3$ -integrin expressing microvessels. Increase in signal intensities due to ultrasound targeted imaging were positively correlated with the frequencies of  $\alpha_v\beta_3$ -integrin expressing microvessels in ovarian tumors at early stage ( $r = 0.72$ ) and late stage ( $r = 0.85$ ). These results support the observations of molecular targeted ultrasound imaging that the detection of  $\alpha_v\beta_3$ -integrin expressing ovarian tumor-associated microvessels can be enhanced *in vivo* by targeted imaging.

#### **Early Detection of OVCA by $\alpha_v\beta_3$ -Integrin-Targeted Ultrasound Imaging in Prospective Study:**

No detectable abnormalities in ovarian morphology were observed by  $\alpha_v\beta_3$ -integrin-targeted ultrasound imaging at 1<sup>st</sup> interval (15 weeks after initial scan) of prospective monitoring of hens. However, at the 2<sup>nd</sup> interval (30 weeks from initial scan) tumor related changes were suspected in 2 of 20 old hens with low egg laying rates. These morphological changes detected by gray scale  $\alpha_v\beta_3$ -integrin-targeted ultrasound imaging included a small solid mass-like appearance limited to a part of the ovary with little or no ascitic fluid (Fig. 4). Sonograms and targeted ultrasound signal intensities of these hens were processed and analyzed off-line immediately. These hens were diagnosed to have early stage OVCA with reference to the signal intensities observed in the

first part of this study (exploratory) described above and euthanized subsequently. Similar changes were observed at the 3<sup>rd</sup> scan (45 weeks from the initial scan) in 6 of the 18 hens. These 6 hens together with the remaining 12 hens with apparently normal ovaries at the end of the 45 weeks were euthanized.

The RI and PI values decreased gradually from 1<sup>st</sup> interval to subsequent intervals (2<sup>nd</sup> and 3<sup>rd</sup> intervals) in hens suspected to have early ovarian tumor related changes (Fig 5). However, such consistent changes were not observed in hens remained normal. The post-targeted RI and PI values were <0.44 and <0.96, respectively for hens predicted to have early stage ovarian tumor related abnormalities. Predictions of targeted imaging were confirmed by gross examination of hens at necropsy and from routine histology. **No abnormality (gross or microscopic) was detected in hens predicted to be normal by sonographic examination.** As predicted by sonography, small solid ovarian mass (n = 8 hens including 2 detected at 2<sup>nd</sup> scan and 6 detected at 3<sup>rd</sup> scan) were limited to a part of the ovary with little or no ascites and histological analysis confirmed the presence of OVCA (3 serous, 4 endometrioid, and 1 mucinous). Thus,  $\alpha_v\beta_3$ -integrin-targeted imaging detected spontaneous ovarian tumors at early stage in hens in the prospective study.

## DISCUSSION

To our knowledge, this is the first study on the enhancement of *in vivo* visualization and early detection of *spontaneous* ovarian tumors by non-invasive transvaginal ultrasound imaging using  $\alpha_v\beta_3$ -integrin-targeted microbubbles in a preclinical animal model. The results of this study demonstrated that  $\alpha_v\beta_3$ -integrin-targeted imaging probes enhanced the visualization of ovarian tumor associated microvessels by transvaginal ultrasound imaging.

Tumor-associated neo-angiogenesis is an early event in ovarian tumor development and represents a potential target for *in vivo* imaging to detect OVCA at early stage<sup>6, 7</sup>. It is known that  $\alpha_v\beta_3$ -integrins is a marker of angiogenic microvessels which is expressed in low levels in quiescent endothelial cells and highly up-regulated during angiogenesis<sup>9, 36</sup>. Over expression of  $\alpha_v\beta_3$ -integrins by the angiogenic endothelium in tumors offers a molecular target for the delivery of imaging agents directly to the tumor vasculature<sup>9, 20</sup>. In the present study, compared with pre-targeted,  $\alpha_v\beta_3$ -integrin-targeted ultrasound imaging agents increased the visualization of ovarian vasculature in normal as well as in tumor bearing ovaries. In ovaries with tumors at the early stage of OVCA, the enhancement was significantly higher than normal hens and increased further in hens with late stages suggesting the efficacy of targeted imaging agents in detecting tumor associated angiogenic microvessels. Similar observations were also reported for induced tumors in mice<sup>9, 13</sup>. Thus  $\alpha_v\beta_3$ -integrin-targeted imaging agent is effective in detecting ovarian tumor associated neoangiogenic microvessels in spontaneous OVCA.

In this study, we assessed the rate of enhancement of visualization of tumor associated microvessels following the injection of  $\alpha_v\beta_3$ -integrin-targeted imaging agents. This study showed that ultrasound signal intensity is correlated with the frequency of  $\alpha_v\beta_3$ -integrin expressing microvessels in the ovary. A significant increase in ultrasound signal intensity following the injection of targeted imaging agent indicates the presence of more angiogenic microvessels in tumor bearing ovaries than normal ovaries. A significantly higher population of  $\alpha_v\beta_3$ -integrin expressing microvessels in tumor bearing ovaries than normal ovaries observed in the immunohistochemical study confirmed ultrasound findings. A similar observation was also reported following injection of targeted imaging agents into mice with induced tumors<sup>9, 13</sup>. Thus



enhanced expression of  $\alpha_v\beta_3$ -integrins was associated with ovarian tumor associated neoangiogenesis and our exploratory study using  $\alpha_v\beta_3$ -integrin-targeted imaging agent enhanced the detection of ovarian tumors in hens. This observation was supported by the results of our prospective studies of monitoring hens in which  $\alpha_v\beta_3$ -integrin-targeted imaging detected ovarian tumors at an early stage. Moreover, consistent decrease in RI and PI values was associated with the development of ovarian tumors. Decrease in RI and PI values indicate decrease resistance to blood flow which might be due to the increased number of angiogenic microvessels associated with tumor development. These data suggests that an imaging approach with  $\alpha_v\beta_3$ -integrins targeted microbubbles may detect spontaneous ovarian tumors at an early stage.

From translational aspects, the results observed in the present study have several unique features. *First*, most of the previous studies used rodent models with induced tumors. In contrast, this study used domestic hens, the only widely available and easily accessible spontaneous model of OVCA. Rodents do not develop spontaneous OVCA and the histopathology of induced OVCA is different than that of spontaneous OVCA. Thus findings from rodent study are difficult to translate to the clinic. *Second*, most of the previous studies with  $\alpha_v\beta_3$ -integrins in induced tumor models used cell lines developed for high expression of  $\alpha_v\beta_3$ -integrins *in vitro* and *in vivo*. Thus they are not suitable to compare with the  $\alpha_v\beta_3$ -integrins expressing tumor endothelial cells in spontaneous ovarian tumors. *Third*, and perhaps most importantly, most of the previous studies used biotin/streptavidin interactions to conjugate targeting ligands. Streptavidin increases non-specific adhesion with endogenous fibronectin<sup>37</sup> and generates anti-streptavidin antibodies in humans<sup>9, 38-40</sup>. In contrast, the covalent conjugation of cRGD peptide used in this study circumvents the potential non-specific adhesion and immunogenicity of streptavidin-based microbubbles and thus provides a significant advantage in targeted imaging and translation to the

clinics. However, this study has also some limitations. One of the main concerns of using  $\alpha_v\beta_3$ -integrin-targeted imaging agent is its specificity for tumor specific microvessels as the angiogenesis (in developing follicles) is a common phenomenon in the ovaries of women of reproductive age. However, as done in this study, the morphology of developing ovarian follicles is very distinct and can be easily detected during gray scale sonography and excluded from the analysis. Additionally, because laying hens do not develop benign ovarian tumors, we did not use animals with benign ovarian tumors. Small sample size specially the number of hens with ovarian tumors may also be a limitation of this study.

In conclusion, the results of this study suggest that the  $\alpha_v\beta_3$ -integrin-targeted imaging probes enhanced the visualization of tumor associated microvessels in the spontaneous ovarian tumors in hens. Our results also suggest that laying hens offer a new platform for studying the feasibility of targeted imaging agents for the detection of OVCA at an early stage. Currently, studies with hens are ongoing in which animals are being monitored prospectively with  $\alpha_v\beta_3$ -integrin-targeted microbubbles together with serum markers to detect spontaneous ovarian tumor development at relatively earlier stages. This animal model of spontaneous OVCA may also be useful for testing the efficacy of different contrast agents *in vivo* in a preclinical setting.

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## Figure Legends

**FIGURE 1.** Enhancement in ultrasound signal intensity from spontaneous ovarian tumors in laying hens following injection of  $\alpha_v\beta_3$ -integrin-targeted imaging agent. A) Pre-targeted gray scale sonogram of a hen ovary suspected to have ovarian solid mass at early stage of ovarian cancer (OVCA). Intensity of the ultrasound signals from the tissue is low and the presence of solid mass is not decisive. B) Gray scale sonogram of the same ovary after the injection of  $\alpha_v\beta_3$ -integrin-targeted imaging agent. Visualization of tumor mass enhanced remarkably (dotted circle). C-D) Ultrasound images showing ovarian tumors at late stage of OVCA with accompanied ascitic fluid (\*). Compared with pre-targeted (C), visualization of the tumor enhanced following the injection of  $\alpha_v\beta_3$ -integrin-targeted imaging agent.

**FIGURE 2.** Gross and histological examination confirmed corresponding  $\alpha_v\beta_3$ -integrins targeted ultrasound predictions of ovarian tumors. Enhancement of visualization of tumor vasculature was not affected by histological sub-types of ovarian tumors. A) Gross appearance of an ovarian

tumor in a hen with stage I ovarian cancer (OVCA) diagnosed in *figure 1(B)*. Tumor was limited to one part of the ovary (red dotted line). B) Histological sub-type (serous) of the same tumor shown in (A). C) Gross ovarian tumor in a hen with late stage OVCA diagnosed in *figure 1(D)*. Tumor mass appeared like cauliflower. D) Histological sub-type (endometrioid) of the same tumor shown in (C) containing back-to-back tumor glands (TG). Hematoxylin & eosin staining. 40X.

**FIGURE 3.** Changes in the expression of  $\alpha_v\beta_3$ -integrins in hen ovaries in association with tumor development and progression.

*Upper panel (A-B):* Immunohistochemical detection of  $\alpha_v\beta_3$ -integrins expressing microvessels in hen ovaries predicted to be normal or to have ovarian tumor by  $\alpha_v\beta_3$ -integrins-targeted imaging.

**A)** Section of a normal laying hen ovary showing expression of few  $\alpha_v\beta_3$ -integrin stained vessels in the follicular theca and stroma beneath the ovarian surface. **B)** Ovarian section of a hen suspected to have ovarian tumor by  $\alpha_v\beta_3$ -integrin-targeted ultrasound imaging. In contrast to (A), many  $\alpha_v\beta_3$ -integrin expressing microvessels are seen in the tumor stroma. G = granulosa layer; T = theca layer of stromal follicle (F); TG, tumor gland. Arrows indicate examples of  $\alpha_v\beta_3$ -integrin expressing microvessels. 40X.

*Bottom panel:* Changes in the frequencies of  $\alpha_v\beta_3$ -integrin expressing ovarian microvessels in association with ovarian tumor development and progression in hens. Compared with normal hens (n=25), the frequency of  $\alpha_v\beta_3$ -integrin expressing microvessels was significantly higher in hens with early stage OVCA (n=7) and increased further in hens at late stage (n=8) of OVCA. Increased number of  $\alpha_v\beta_3$ -integrin expressing microvessels confirms the higher ultrasound signal intensities in hens with ovarian tumors than normal hens. Each bar represents mean  $\pm$  SD of the

frequency of  $\alpha_v\beta_3$ -integrins expressing microvessels and bars with different letters are significantly different.

**FIGURE 4.** Detection of spontaneous ovarian tumors at early stage by  $\alpha_v\beta_3$ -integrin-targeted ultrasound imaging in hens in a prospective study. Hens with no detectable ovarian abnormality were monitored by  $\alpha_v\beta_3$ -integrin-targeted ultrasound imaging for the development of ovarian cancer (OVCA) for 45 weeks. A) Pre-targeted gray scale sonogram of a hen ovary. Detection of solid mass was difficult because of low ultrasound signal intensity. B) Post-targeted gray scale sonogram of the same ovary shown in (A). Enhanced signal from post-targeted gray scale ultrasound imaging detected the presence of a tumor in a part of the ovary (red dotted circle). C) Gross ovarian tumor at early stage diagnosed in (B). Tumor mass was limited to a part of the ovary (red dotted circles). See the text for detail description on the prospective monitoring and OVCA development.

**FIGURE 5.** Changes in ultrasound resistive indices (RI) in association with ovarian tumor development in laying hens displayed as box and whiskers. The median, range (whiskers), and 25th to 75th percentiles (box) are shown ( $n = 20$  hens in each group). Hens were monitored prospectively by  $\alpha_v\beta_3$ -integrins targeted ultrasound imaging at 15 weeks interval. RI values in hens (that developed OVCA later) reduced consistently ( $P < 0.001$ ) from 1<sup>st</sup> scan to 3<sup>rd</sup> scan at the time of OVCA diagnosis (left panel, an imaginary dotted line shows the gradual reduction in RI values). Reduced RI values indicate lower resistance leading to the increased flow of blood to the tissue, a characteristic feature associated with tumor development. Similar patterns of change

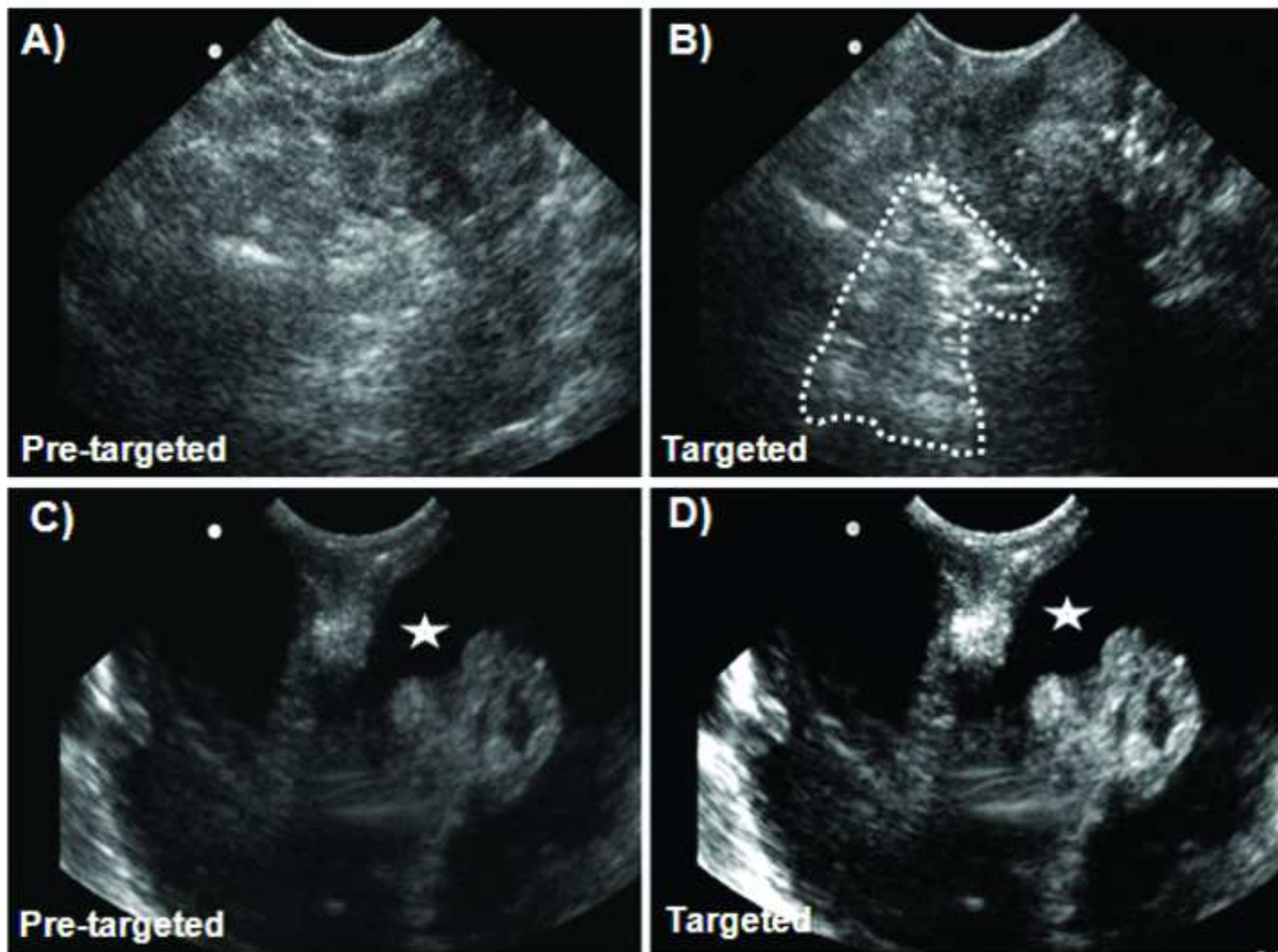
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560 were also observed for PI values. However, such consistent changes in RI values were not  
561 observed in hens remained normal and did not develop OVCA (left panel). Bars with different  
562 letters indicate significant differences among the different scanning intervals within the same  
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Figure

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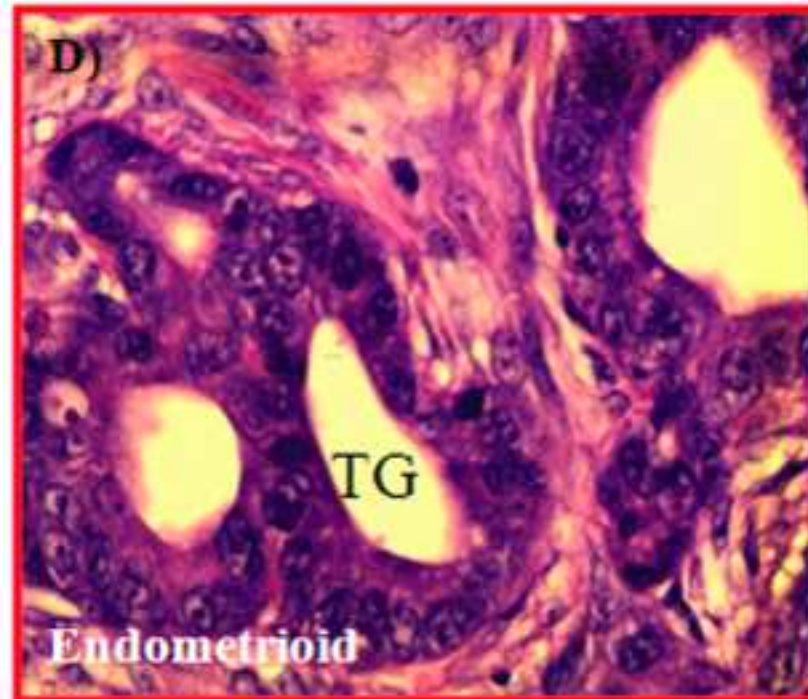
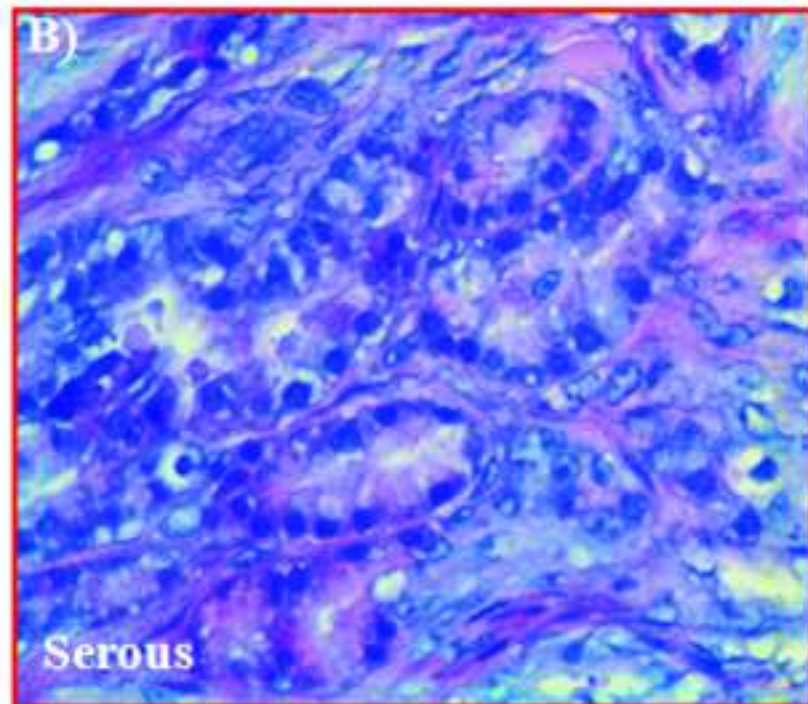
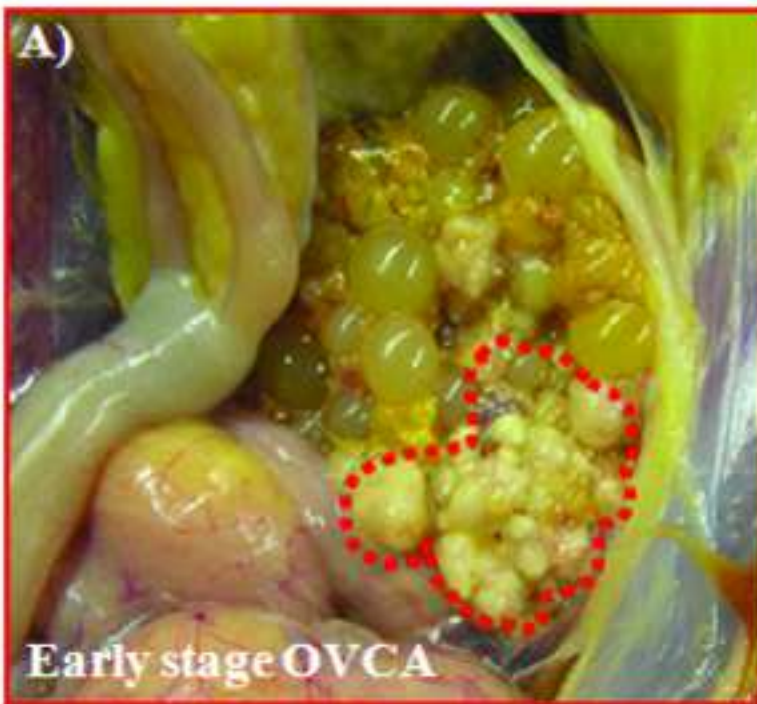
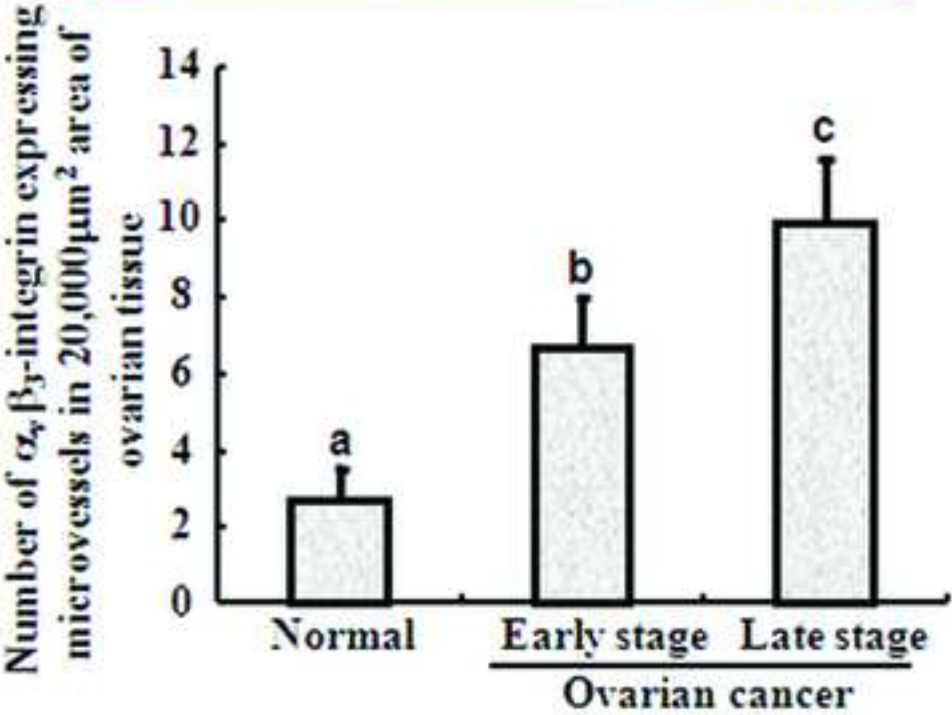
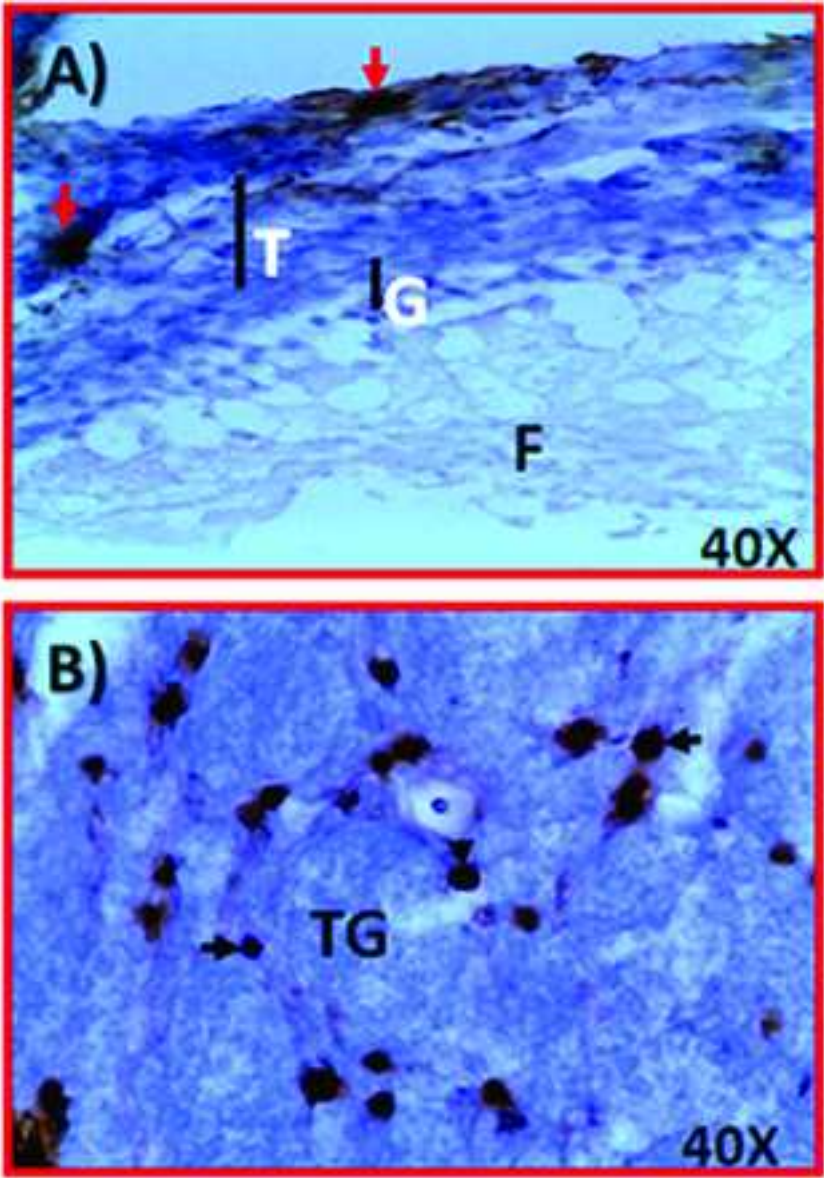


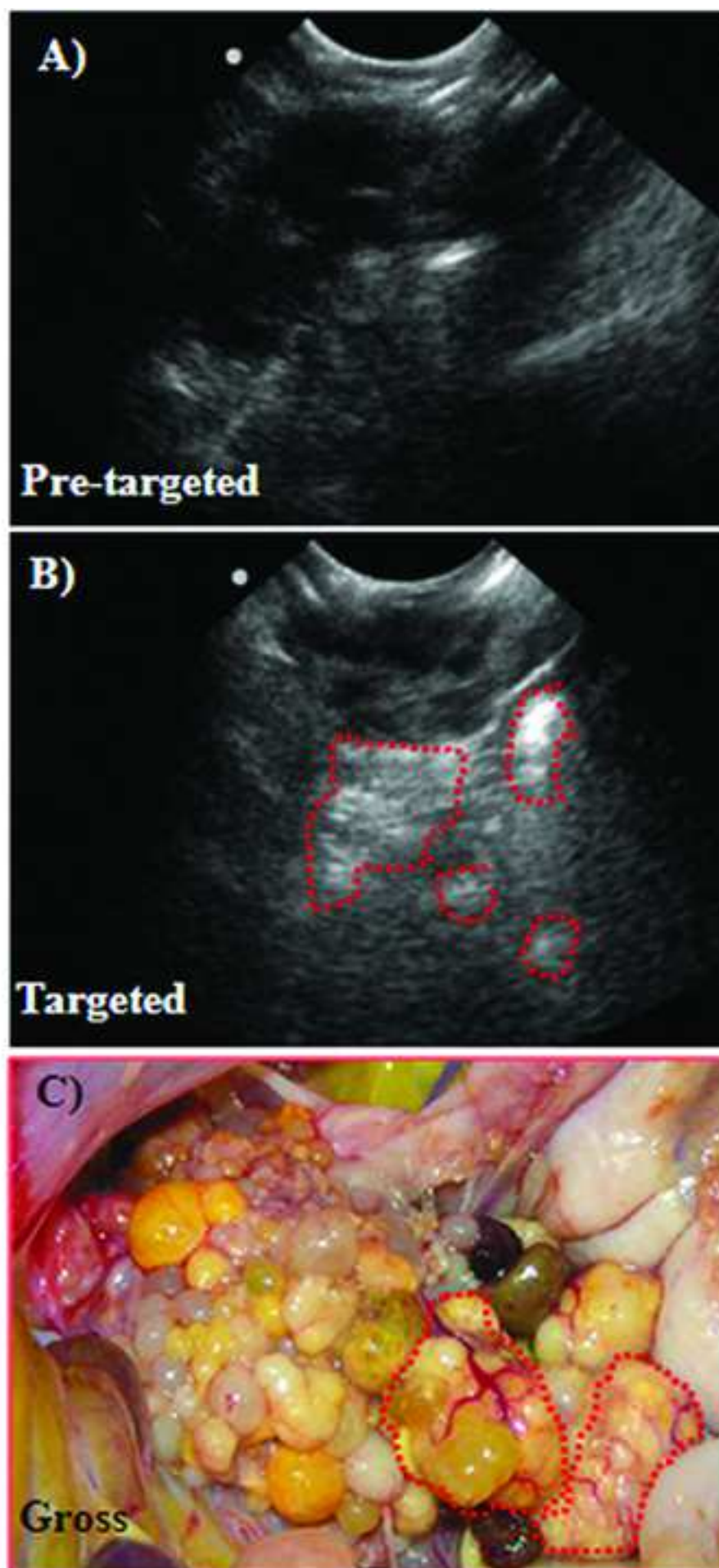
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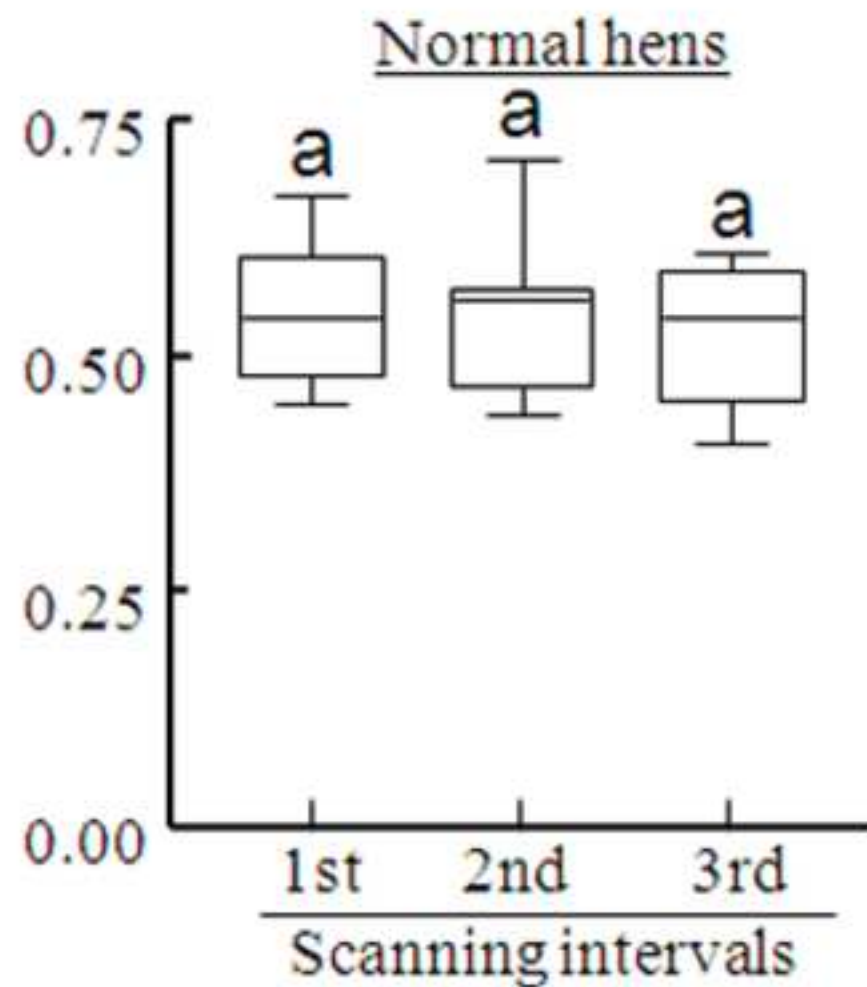
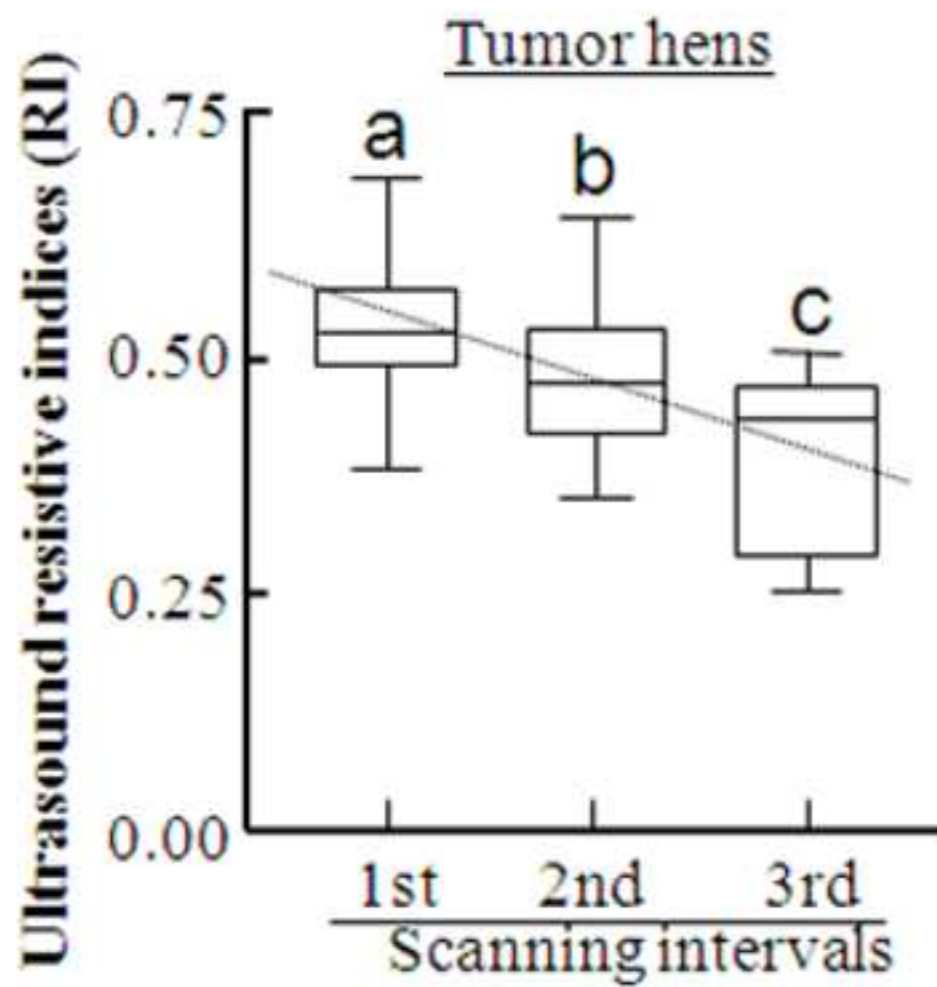
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## Expression of Death Receptor 6 by Ovarian Tumors in Laying Hens, a Preclinical Model of Spontaneous Ovarian Cancer<sup>1</sup>

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### Abstract

Tumor-associated neoangiogenesis and suppression of antitumor immunity are hallmarks of tumor development and progression. Death receptor 6 (DR6) has been reported to be associated with suppression of antitumor immunity and tumor progression in several malignancies. However, expression of DR6 by malignant ovarian epithelial tumors at an early stage is unknown. The goals of this study were to determine whether DR6 is expressed by malignant ovarian epithelial tumors at an early stage and to examine whether DR6 expression is associated with ovarian cancer (OVCA) progression in a laying hen model of spontaneous OVCA. Expression of DR6 was examined in normal and malignant ovaries, normal ovarian surface epithelial (OSE) cells, or malignant epithelial cells and in serum of 3-year-old hens. The population of microvessels expressing DR6 was significantly higher in hens with early-stage OVCA than hens with normal ovaries ( $P < .01$ ) and increased further in late-stage OVCA. The results of this study showed that, in addition to microvessels, tumor cells in the ovary also express DR6 with a significantly higher intensity than normal OSE cells. Similar patterns of DR6 expression were also observed by immunoblot analysis and gene expression studies. Furthermore, DR6 was also detected in the serum of hens. In conclusion, DR6 expression is associated with OVCA development and progression in laying hens. This study may be helpful to examine the feasibility of DR6 as a useful surrogate marker of OVCA, a target for antitumor immunotherapy and molecular imaging and thus provide a foundation for clinical studies.

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### Introduction

Ovarian cancer (OVCA) is the most lethal tumor among gynecologic malignancies, with an estimated yearly incidence rates of 22,000 in the United States and 42,000 in Europe [1,2]. In most cases, OVCA is detected at advanced stages, and despite the remarkable improvements in treatment strategies, most of these patients have recurrences. The reasons for failure to detect and treat OVCA at an early stage as well as its high rate of recurrences are the lack of an effective early detection test, suppression of antitumor immunity by the tumor, and resistance to drugs [3–5]. OVCA differs from other malignancies in its specific

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dissemination pattern, which is characterized by tumor spread in a diffuse intrapelvic and abdominal manner [5]. Thus, the local tumor microenvironment including tumor-associated neoangiogenesis and suppression of antitumor immunity play important roles in the development and progression of ovarian tumors. However, the way tumor establishes neoangiogenesis and escapes antitumor immune surveillance is not well understood. Information on factors related to the development of tumor-associated angiogenesis and immune suppression in the tumor microenvironment is important because it may offer opportunities to establish an early detection test as well as targeted antitumor therapy. Death receptor 6 (DR6) has been suggested to be one of such factors because of its expression by blood vessels and its involvement in immunoregulation [6,7].

DR6 is a member of the tumor necrosis factor  $\alpha$  receptor superfamily (TNFRSF21) [8,9]. Although DR6 has been shown to be involved in apoptotic cell death, elevated expression of DR6 has been observed in several tumors in humans [10]. DR6 expression was increased in tumor tissues from patients with late-stage prostate and breast cancers compared with its level in normal tissues [10]. Recently, DR6 concentration in the serum has been shown to be elevated in patients with late-stage OVCA [6]. In addition, DR6 has been demonstrated to be expressed by blood vessels in tumor tissues [11]. All these reports suggest that increased DR6 expression is associated with advanced stages of several malignancies including OVCA. However, its association with early-stage OVCA including tumor-associated angiogenesis is not known.

Suppression of antitumor immunity has been suggested as one of the mechanisms of tumor survival and progression [5]. Despite the presentation of antigens by malignant cells, which should induce immune-mediated rejection, spontaneous rejection of established tumor is rare [5]. Inefficient tumor rejection by the immune system is not only a passive result of insufficient effector cells [12,13] because tumors induce immune-suppressive mechanisms that protect them against eradication [3,4]. Compared with other solid tumors, studies on immunosuppression by ovarian tumors are very few. As in other epithelial malignancies, antitumor immune responses were reported to be elicited against ovarian tumors [14–16], but these responses were not effective enough to eliminate bulky tumor [5]. Moreover, the distinctive type of disease dissemination (peritoneal spread and metastasis) makes OVCA unique compared to other solid tumors. Although the precise mechanism(s) of inadequate or defective antitumor immune responses are not well understood, expression or secretion of immunosuppressive factors by the tumor has been suggested as a potential strategy for immune evasion. DR6 has been reported to alter normal differentiation of monocyte to immature dendritic cells rather than mature dendritic cells [17] and immature dendritic cells have been demonstrated to induce tolerance [18]. Furthermore, because of its inhibitory roles in T- and B-cell proliferation and migration, DR6 has been proposed to be immunosuppressive and may be involved in tumor cell survival and immune evasion [7]. However, expression of DR6 by ovarian tumors at early stages as well as inhibition of antitumor immunity by DR6 in OVCA patients is not known.

To develop and improve the efficacy of an antitumor immunotherapy, more insight into the interaction between ovarian cancer (OVCA) and the immune system is needed. Information on DR6 expression by ovarian tumors may lead to the identification of additional targets, which may allow opportunities for developing new therapeutic approaches to inhibit tumor progression. Studies on the

immune response against tumors or immune suppression by ovarian tumors at early stages are lacking. Furthermore, if DR6 is expressed by ovarian tumor-associated neoangiogenic microvessels, it may be a useful target for the early detection of OVCA by Doppler ultrasound imaging. The difficulty in identifying patients with OVCA at an early stage and the limited access to tumor tissue are significant barriers to the study and to the development of an effective immunotherapy against OVCA. Laying hens are the only widely available and easily accessible animals that develop OVCA spontaneously with high incidence rates and remarkably similar histologic subtypes and tumor markers to human OVCA [19–21]. In addition, avian DR6 has been reported to be orthologous to human DR6 (70% homology) and is expressed by the hen ovaries [22]. Thus, the objective of the present study was to explore whether DR6 is expressed by ovarian tumors in hens and, if so, whether its expression changes in association with the stage of the tumors and histologic subtypes.

## Materials and Methods

### Animals

A flock of 3-year-old commercial strains of White Leghorn laying hens (*Gallus domesticus*) was maintained under standard poultry husbandry practices. Hens ( $n = 120$ ) were selected based on their egg laying rates (normal or low) and transvaginal ultrasound scanning as reported previously [23]. The incidence of OVCA in hens of this age group is approximately 15% to 20% and is associated with low or complete cessation of egg laying [19,23]. All experimental procedures were performed according to the institutional animal care and use committee-approved protocol.

### Tissue Collection and Processing

**Serum samples.** Blood was obtained from brachial veins of all hens before euthanasia and centrifuged (1000g for 20 minutes), and serum samples were stored at  $-80^{\circ}\text{C}$ .

**Ovarian morphology and histopathology.** Ovarian pathology and tumor staging were performed by gross and histologic examination as reported previously [19]. Each normal and malignant ovary (tumor bearing ovary) was divided into four portions for protein extraction, total RNA collection, paraffin and frozen embedding for routine histology, and immunohistochemical studies as reported previously [24]. Normal ovarian surface epithelial (OSE) cells or tumor cells (malignant epithelial cells of the tumor) in hens with OVCA were collected as reported earlier [25,26]. Samples were divided into three groups including normal and early- and late-stage OVCA based on gross inspection and microscopy as previously reported [19].

### Preparation of Ovarian Specimen for Biochemical Analysis

Snap-frozen ovarian tissues as well as normal OSE cells and tumor cells from hens with normal ovaries or hens with OVCA were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) as reported previously [27], were centrifuged; the supernatant was collected and the protein content of the extract was measured and stored at  $-80^{\circ}\text{C}$ .

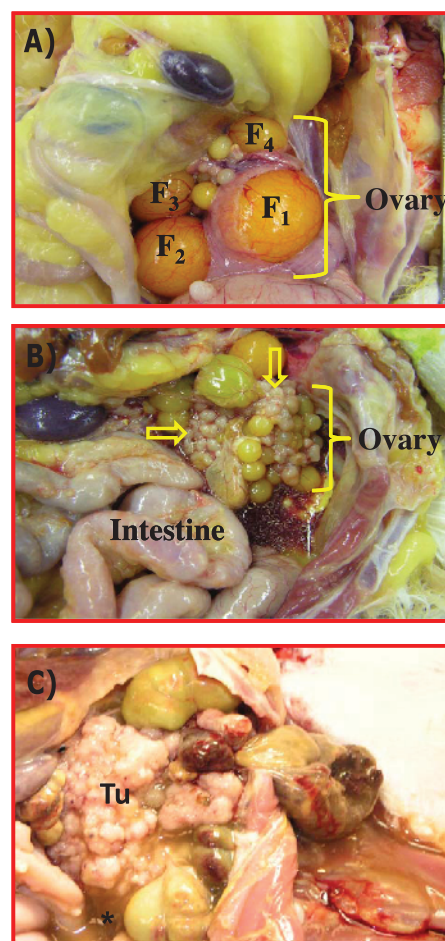
### Immunohistochemistry

Rabbit polyclonal anti-chicken DR6 antibodies were used as primary antibodies, and immunoreactions were determined using Vectastain Elite ABC kit (Universal, RTU; Vector Laboratories, Inc, Burlingame, CA). Normal or malignant ovaries ( $n = 15$  hens each for normal, early, and late stages) were selected randomly for immunohistochemical study. The number of hens for each group for immunohistochemistry was determined based on the power analysis to achieve significant differences in different parameters among the normal or malignant groups. Briefly, after deparaffinization, antigens on the sections were unmasked by heat treatment, endogenous peroxidase in the sections was inactivated, and nonspecific staining was blocked by incubating with 0.3% hydrogen peroxide in methanol and normal horse serum, respectively. Sections were then incubated for 2 hours with primary antibodies (1:100 dilution) followed by 1 hour of incubation with secondary antibodies (Vectastain Elite ABC kit; Vector Laboratories). Immune reaction products on the sections were visualized by incubating with diaminobenzidine and hydrogen peroxide mixture (DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine; Vector Laboratories). Sections were then counterstained with hematoxylin, dehydrated, and covered. Control staining was carried out simultaneously in which the first antibodies were omitted and normal serum was used. No staining was found in these control slides.

Sections were then examined under a light microscope attached to digital imaging software (MicroSuite version 5; Olympus Corporation, Tokyo, Japan). The population of microvessels expressing DR6 as well as the intensity of DR6 staining by the normal ovarian stroma or stroma (around the tumor) in malignant ovaries was determined. Three sections per ovary and five regions of interest with the highest immunoreactivity ( $20,000 \mu\text{m}^2$  per region at an objective of  $\times 40$  and ocular magnification of  $\times 10$ ) per section were selected. Using the software, the intensity of the DR6 immunostaining in each region was measured and recorded as pixel values in  $20,000 \mu\text{m}^2$  of the section as reported previously [28]. The mean of pixel values of these five regions in a section was considered as the intensity of DR6 in a  $20,000\text{-}\mu\text{m}^2$  area of each section. The mean intensity of three sections was considered as the DR6 staining intensity in a  $20,000\text{-}\mu\text{m}^2$  area of each normal or malignant ovary. The groupwise DR6 staining intensity (normal or tumor groups) was expressed as mean  $\pm$  SD in a  $20,000\text{-}\mu\text{m}^2$  area of ovaries in normal or malignant groups. Similarly, and using the same software, the population of microvessels expressing DR6 in the section was counted and reported as the frequency (mean  $\pm$  SD) of DR6-expressing microvessels in a  $20,000\text{-}\mu\text{m}^2$  area of the stroma of normal or malignant ovaries as reported previously [29].

### One-dimensional Western Blot

Ovarian expression of DR6 was confirmed by immunoblot analysis using homogenates of normal ( $n = 5$ ) or malignant ovaries as well as normal OSE cells or tumor cells. Twelve samples (four from each of the serous, endometrioid, and mucinous samples) at early and late stages of OVCA were selected for immunoblot analysis based on their immunoreactivity for DR6 in immunohistochemistry. Immunoreactions on the membrane were visualized as a chemiluminescence product (Super Dura West substrate; Pierce/Thermo Fisher, Rockford, IL), and the image was captured using a Chemidoc XRS (Bio-Rad, Hercules, CA). Similarly, serum samples from the same hens used for ovarian DR6 expression were selected for immunoblot analysis to examine the presence of DR6 in serum. Serum samples were filtered by acetonitrile and chloroform-methanol precipitation before using for immunoblot analysis [27].



**Figure 1.** Gross morphology of normal and malignant ovaries in laying hens. (A) Fully functional normal ovary in a laying hen. Laying hens ovulate and subsequently lay eggs once a day for 5 or 6 days in a week in a continuous manner and then take a pause for 1 day before laying resumes. Thus, the ovary in a fully functional laying hen contains a set of multiple large and growing preovulatory follicles arranged in a hierarchy of sizes (F1-F5). The largest follicle (F1) is destined to ovulate soon and then the second largest follicle (F2) becomes F1 and a small growing follicle is recruited from the ovarian stroma to the hierarchy to maintain the laying rates. (B) Ovarian tumor at an early stage in a hen showing solid tumor mass (arrows) limited to the ovary. (C) Ovarian tumor at a late stage. The tumor (Tu) appears like a cauliflower and has metastasized to other organs with accompanied profuse ascites (\*).

### Reverse Transcription–Polymerase Chain Reaction

DR6 mRNA expression was assessed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) as reported previously [30]. For RT-PCR analyses, serum and tissue samples from 5 hens with normal ovaries, from 12 hens with early stages of OVCA (4 for each histologic subtype), and from 12 hens with late stages of OVCA (4 for each histologic subtype) were selected based on their reactivity in immunohistochemistry and immunoblot analysis. Hen-specific DR6 primers were designed by OligoPerfect Designer software (Invitrogen, Carlsbad, CA) using the DR6 sequence from the National Center for Biotechnology Information (accession no. A1980074) as reported earlier [22]. The forward primer was 5'-GAT GGA GGA CAC CAC GCC-3' and the reverse primer was 5'-TCG GGG TTG AGG ATG TGC-3'.  $\beta$ -Actin was used as the endogenous control, with a forward primer of



TGCGTGACATCAAGGAGAAG and a reverse primer of ATGC-CAGGGTACATTGTGGT. The expected base pair size for the DR6 amplicon was 384 bp and that for  $\beta$ -actin was 300 bp. PCR amplicons were visualized in a 3% agarose gel (Pierce/Thermo Fisher) in Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide. The image was captured using a ChemiDoc XRS system (Bio-Rad).

### Statistical Analysis

The differences in the intensity of DR6 immunostaining and the number of microvessels expressing DR6 in normal ovaries and malignant ovaries were assessed by analysis of variance, *F* tests, and the alternative nonparametric Kruskal-Wallis tests. Subsequently, pairwise comparison between the groups (normal and early- and late-stage OVCA) by two-sample *t* tests and alternative Mann-Whitney tests were performed. All reported *P* values are two-sided, and *P* < .05 was considered significant. Statistical analyses were performed with SPSS (PASW) version 18 software (IBM, Inc, Armonk, NY).

## Results

### Ovarian Morphology

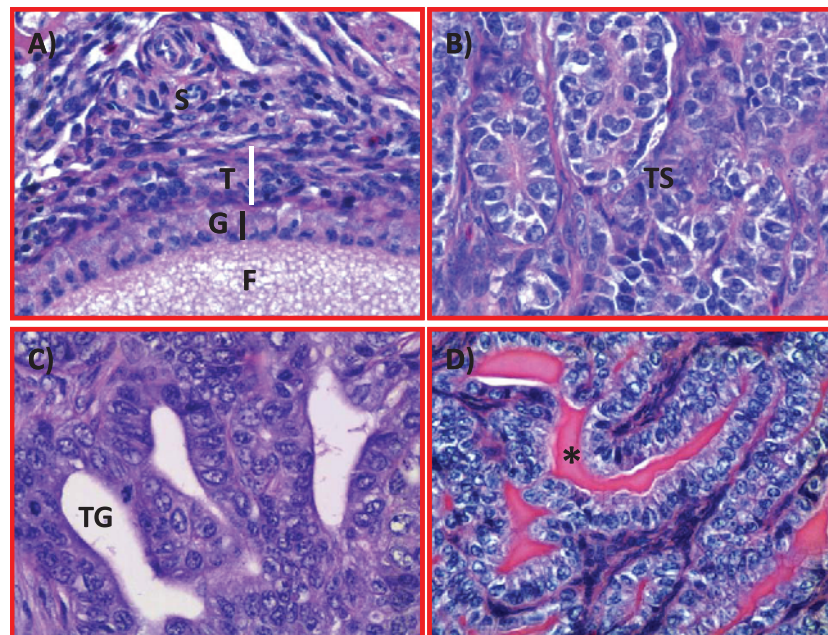
In laying hens, only the left ovary becomes functional, and the rate of egg production declines with aging. A fully functional ovary contains five to six developing large preovulatory follicles (Figure 1A). As the hen ages, the rate of egg production decreases. The ovary of an older (>3 years old) healthy hen with a low rate of egg production contains

fewer than three large preovulatory follicles. In apparently healthy hens that have stopped laying eggs, the ovaries were atrophied and the oviducts were smaller. Solid tissue masses either limited to a small part or the entire ovary, with or without ascites, were observed in 12 hens. These hens were diagnosed with early-stage OVCA (Figure 1B). In 16 hens, tumor had metastasized to the abdominal organs with moderate to profuse ascites. These hens were diagnosed with late-stage OVCA (Figure 1C).

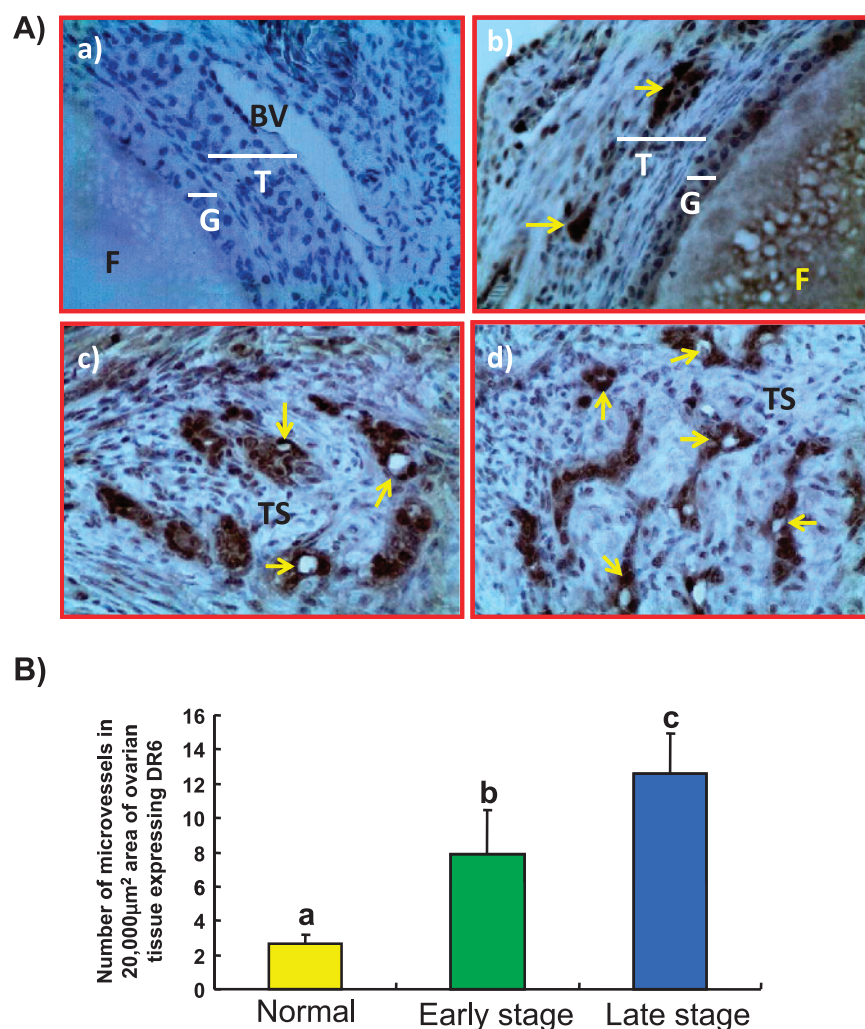
**Histopathology.** Cortical follicles with or without distinguishable granulosa cell and theca layer were embedded in the ovarian stroma of hens with normal ovaries (Figure 2A). Tumors were confirmed in all hens displaying gross ovarian solid masses (12 hens at early stage with solid masses limited to the ovaries or in 16 hens with late-stage OVCA) by routine histology (Figure 2, B-D). However, tumor-related microscopic changes (including focal lesions containing large cells with irregular shapes and pleomorphic nuclei) were also found during histologic examinations in 11 additional hens that had no gross ovarian tumor and were grouped in early-stage OVCA. Thus, a total of 23 (12 + 11) hens had early-stage OVCA, 16 had late-stage OVCA, whereas 81 hens had normal ovaries. Tumors were typed as serous (*n* = 17), endometrioid (*n* = 12), mucinous (*n* = 8), clear cell (*n* = 1), as well as mixed (*n* = 1, seromucinous) as reported previously [19].

### Tissue Expression of DR6

Microvessels expressing DR6 were detected in both normal and malignant ovaries (Figure 3). Most of the DR6-expressing ovarian



**Figure 2.** Microscopic features of hen ovaries. Paraffin-embedded sections from normal or malignant ovaries with tumors were stained with hematoxylin and eosin. (A) Section of a normal ovary showing a developing follicle embedded in the ovarian stroma. (B) Section of an ovarian serous carcinoma showing a solid sheet of tumor surrounded by fibromuscular tissue. The tumor contains a labyrinth of slitlike glandular spaces lined by cells with large pleomorphic nuclei and mitotic figures. (C) Section showing endometrioid carcinoma displaying confluent back-to-back glands. Glands contain a single layer of epithelial cells with sharp luminal margins. (D) Section of a mucinous carcinoma. Glands in clusters with scarce intervening stroma lined by columnar and goblet cells with intracytoplasmic mucin. Original magnification,  $\times 40$ .



**Figure 3.** (A) Immunohistochemical detection of DR6-expressing microvessels in hen ovaries with or without tumor. (a) Section of a normal ovarian stroma immunostained by omitting primary antibodies used as control. No immunopositive vessel is seen. (b) Serial section from the same normal ovary immunostained with primary antibodies. Very few DR6-expressing vessels are seen. (c) An ovarian section from a hen with early-stage OVCA. Compared to the normal ovary, many DR6-expressing microvessels are seen in the stroma between tumors. (d) Section of a malignant tumor from a hen with late-stage OVCA. Many DR6-expressing microvessels are localized in the tumor stroma. BV indicates blood vessel; F, follicle; G, granulosa layer; T, theca layer; TS, tumor stroma. Arrows indicate DR6-expressing microvessels. Original magnification,  $\times 40$ . (B) Changes in the frequency of DR6-expressing ovarian microvessels relative to ovarian tumor development and progression in hens. The frequency of microvessels expressing DR6 in a 20,000- $\mu\text{m}^2$  area of normal ( $n = 15$ ) and malignant ovaries (expressed as the mean  $\pm$  SD,  $n = 15$  each for early and late stages). Compared to the normal ovary, the frequency of microvessels expressing DR6 was significantly ( $P < .01$ ) higher in hens with early-stage OVCA cancer and increased further ( $P < .001$ ) as the disease progressed to a late stage in hens. Each bar with a different letter indicates significant differences ( $P < .001$ ) between normal and tumor groups.

microvessels in hens with normal ovaries had thick, complete, and continuous vessel walls with intense staining. These vessels were located in the theca layer of the follicles and a few vessels were in the ovarian stroma. In contrast, most of the DR6-expressing vessels in malignant ovaries were discontinuous or incomplete with thin vessel walls (Figure 3A). The number of DR6-expressing microvessels was significantly higher ( $P < .01$ , exact Mann-Whitney test) in hens with early (mean  $\pm$  SD =  $8.0 \pm 2.29$  microvessels per 20,000- $\mu\text{m}^2$  area of stroma) and late ( $13.0 \pm 2.37$  microvessels per 20,000- $\mu\text{m}^2$  area of stroma in malignant ovaries) stages of OVCA than in hens with normal ovaries ( $3.0 \pm 0.52$  microvessels per 20,000- $\mu\text{m}^2$  area of stroma in normal ovaries; Figure 3B). However, significant differences in the

population of DR6-expressing microvessels among the histologic subtypes were not observed.

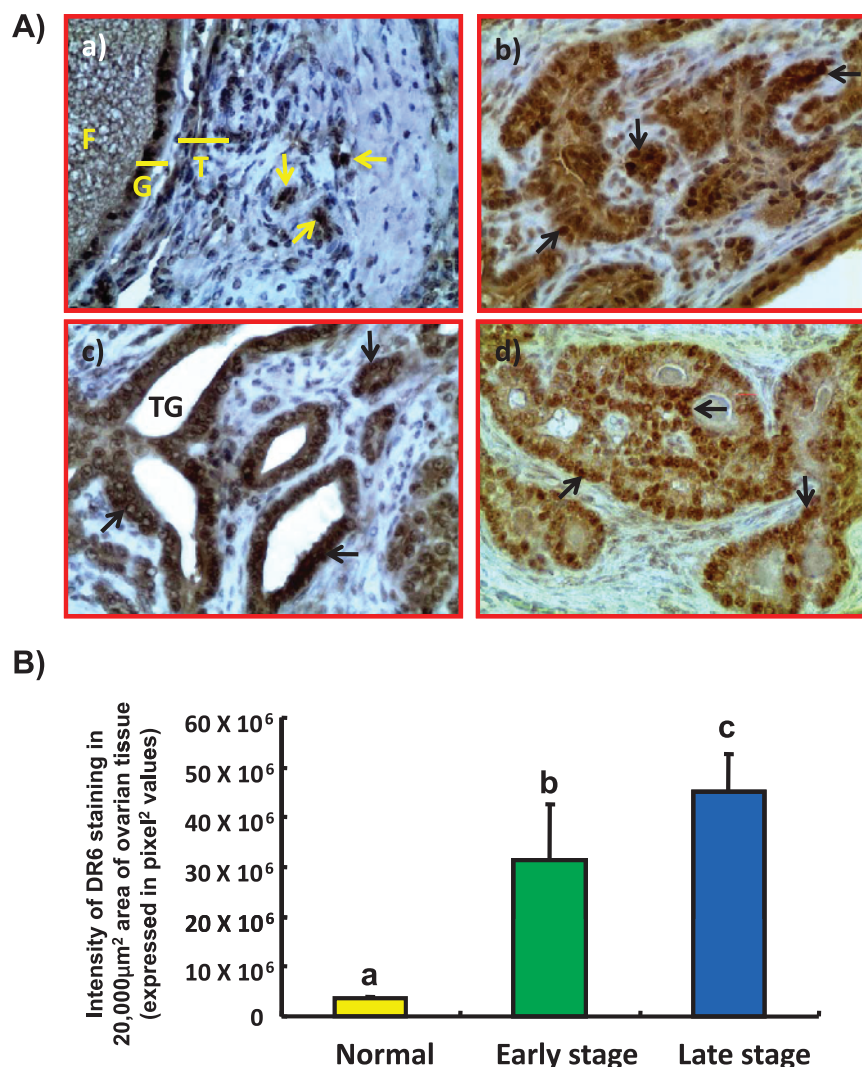
Tumor cells (Figure 4A) as well as a few normal OSE cells expressed DR6. In addition, rounded (T lymphocyte like) to irregularly shaped (macrophage-like) DR6 cells were also detected in the stroma of the normal ovaries or malignant ovaries. Compared with normal ovaries (mean  $\pm$  SD =  $3.6 \times 10^5 \pm 3.8 \times 10^5$  in a 20,000- $\mu\text{m}^2$  area), the intensities of DR6 staining increased approximately 9-fold ( $P < .001$ ) in hens with early-stage OVCA and 13-fold ( $P < .001$ ) in hens with late-stage OVCA, respectively (Figure 4B). However, significant differences in DR6 staining intensities were not observed among three different histologic subtypes of OVCA in hens.



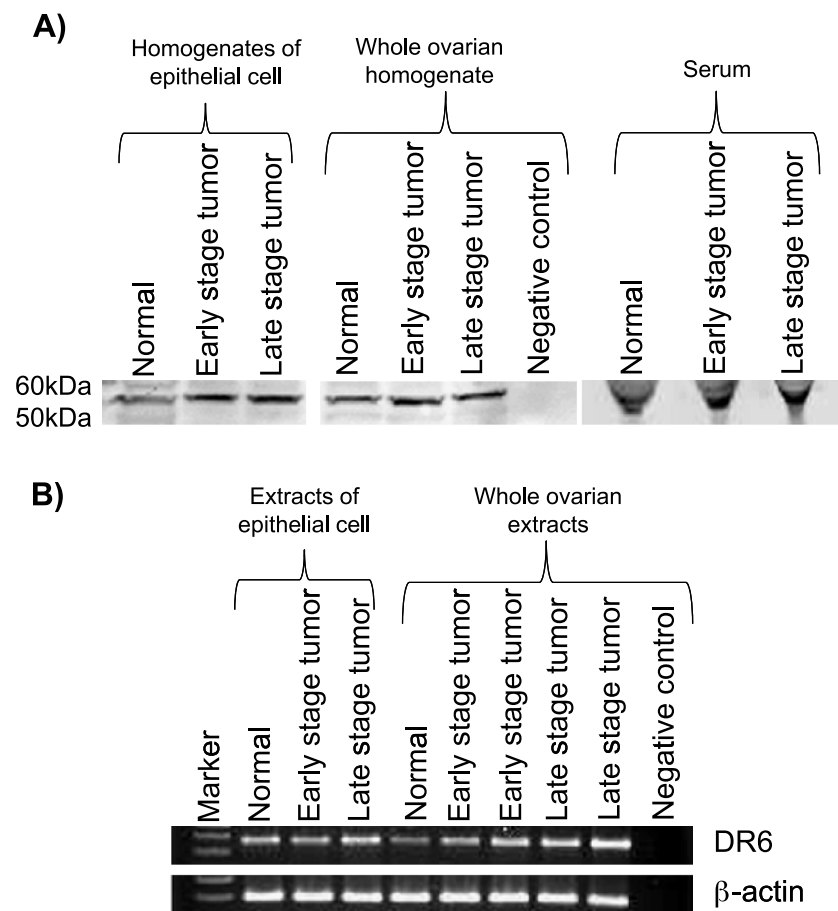
**Immunoblot analysis for DR6 protein in ovarian tissues and serum samples.** Immunohistochemical expression of DR6 in normal or malignant ovaries was confirmed by immunoblot analysis using homogenates of OSE cells (from normal ovaries) or tumor cells (from malignant ovaries) as well as homogenates from whole normal or malignant ovaries. A band of 50 to 60 kDa was detected in the homogenates of cells (normal OSE cells or tumor cells) and tissues (whole normal or tumor ovaries; Figure 5A). In addition, immunoreactive 50- to 60-kDa DR6 protein was also detected by immunoblot analysis in the serum of hens with normal ovaries or those with OVCA (Figure 5A). Compared with the whole ovarian homogenates or serum from hens with normal ovaries, immunoreactivity for DR6 protein was intense in the homogenates of malignant ovaries or serum from hens with OVCA (Figure 5A). These results support the immunohistochemical observation that OSE in hens with normal ovaries and tumor

cells in hens with OVCA express DR6 protein. Moreover, these epithelial cells may be a source of DR6 proteins in the circulation of laying hens because DR6 was detected in the serum of hens with normal ovaries or those with OVCA.

**Expression of DR6 messenger RNA.** DR6 messenger RNA (mRNA) expression confirmed the observed variations in ovarian DR6 expression among hens with normal ovaries or those with OVCA. Although the patterns of DR6 mRNA expression were similar between normal OSE and tumor cells from early-stage OVCA, it was stronger for tumor cells from late-stage OVCA. Compared with the hens with normal ovaries, strong amplification of signal for DR6 (Figure 5B) was observed in the ovarian extracts from hens with early-stage OVCA and the amplification was stronger in hens with late-stage OVCA. However, differences in DR6 mRNA expression were



**Figure 4.** (A) Expression of DR6 by tumor cells in malignant ovaries in hens. (a) Section of a normal ovary immunostained for DR6 expression. Very few immunopositive cells are present in the ovarian stroma. (b-d) Sections of different histologic subtypes of OVCA in hens including serous (b), endometrioid (c), and mucinous (d) at early stages immunostained for DR6 expression. Compared with the normal OSE cells, tumor cells in all three histologic subtypes stained intensely. Original magnification,  $\times 40$ . (B) Changes in the DR6 staining intensity relative to ovarian tumor development and progression in hens. The intensity of DR6 staining is expressed as the pixel values (mean  $\pm$  SD) in a 20,000- $\mu\text{m}^2$  area in normal ovarian stroma ( $n = 15$ ) or in malignant ovaries. Compared to the normal ovary, the intensity of DR6 staining was significantly ( $P < .001$ ) higher in hens with early-stage OVCA ( $n = 15$ ) and increased further ( $P < .001$ ) in late-stage OVCA ( $n = 15$ ). Each bar with different letter indicates significant differences ( $P < .001$ ) among normal or OVCA stages.



**Figure 5.** Immunoreactive (A) DR6 protein or (B) mRNA in serum and ovaries of hens with normal ovaries or those with OVCA. (A) One-dimensional Western blot analysis: Immunoreactive DR6 proteins of 50- to 60-kDa molecular weight were detected in the homogenates of normal OSE cells or tumor cells, in homogenates of whole normal or malignant ovaries, as well as in serum of hens by one-dimensional Western blot. Compared to the hens with normal ovaries, relatively stronger immunoreactive bands for DR6 proteins were observed in serum and ovaries of hens with early and late stages of OVCA. No immunoreactive band was detected in the negative control in which protein sample was omitted. (B) Semiquantitative RT-PCR: mRNA expression for DR6 was detected in the extracts of normal OSE cells or tumor cells and in extracts of normal and malignant ovaries by semiquantitative PCR. Compared to the weak expression by normal ovaries and OSE, strong amplification for DR6 mRNA was observed in the extracts of malignant ovaries and tumor cells in hens with early- and late-stage OVCA. No DR6 mRNA expression was detected in the negative control in which mRNA sample was omitted.

not observed among different histologic subtypes of OVCA at the same stage (early or late). Overall, compared to hens with normal ovaries, strong amplification of DR6 mRNA was observed in hens with OVCA as observed for immunoreactivities in immunohistochemistry and immunoblot analysis.

## Discussion

This is the first report on the expression of death receptor (DR6) by tumor cells of malignant ovaries in hens. The expression of DR6 was significantly higher in the tumor cells of malignant ovaries than OSE cells of normal ovaries. Furthermore, the population of ovarian microvessels expressing DR6 was significantly higher in hens with early-stage OVCA than hens with normal ovaries and increased further in hens with late-stage OVCA. In addition, DR6 was also detected in the serum of hens. Thus, the results of the present study suggest that the increase in DR6 expression may be associated with ovarian tumor development and progression in laying hens.

Tumor-associated neoangiogenesis (TAN) and suppression of anti-tumor immunity are two of the early events required for the survival

and progression of the tumor. Increased numbers of immature microvessels with disorganized and discontinuous smooth muscle layers are the characteristic features of ovarian TAN in patients as well as in laying hens with OVCA [24]. Compared to hens with normal ovaries, the number of DR6-expressing microvessels was significantly higher in hens with early-stage OVCA and increased further in hens with late-stage OVCA. A recent study has reported increased expression of DR6 by ovarian tumors in patients with advanced-stage OVCA [6]. In this study, the population of DR6-expressing ovarian microvessels increased significantly at an earlier stage even before the tumor became grossly detectable. Recently, DR6 was reported to be required for angiogenesis in the central nervous system [31]. Although precise reason(s) for the increase in the population of DR6-expressing microvessels in malignant ovaries is not known, it is possible that DR6 will play a role in the development of ovarian TAN.

Despite the presence of an antitumor immune response [5,32], the rare eradication of ovarian tumors and their progression suggest that multiple mechanisms are used by the tumor to escape immune rejection. The expression of DR6 has been reported to be increased significantly



in cell lines and patients with prostate and breast cancers [7,10,33]. In the absence of DR6, ligation of the T-cell receptor results in enhanced T-cell proliferation, activation, and skewed  $T_H2$  cytokine production. Similarly, B cells lacking DR6 show increased proliferation, cell division, and cell survival on mitogenic stimulation (anti-CD40 and LPS) or BCR ligation. DR6 $^{-/-}$  mice showed increased  $T_H2$  immune responses to both T-dependent and -independent antigens. In contrast, it is suggested that increased DR6 expression on tumor cells results in the cleaving of extracellular part of DR6 from the cell surface by matrix metalloproteinase 14. This shed DR6 reported to attenuate the *in vitro* differentiation of monocytes into immunotolerant instead of immunocompetent dendritic cells, which can contribute to tumor evasion from the immune system [17]. All these reports indicate that DR6 plays important roles in imparting tolerance to local immune response. In the present study, the expression of ovarian DR6 was significantly high in hens with early-stage OVCA than in hens with normal ovaries and increased further in hens with late-stage OVCA. Although the significance of increased DR6 expression by malignant cells is not known, the results of the current study suggest that increased DR6 expression may play important roles in the suppression of immunity against ovarian tumors.

The results of the current study suggest several translational significances. Because the malignant tumors in hens express DR6 and it is also present in the serum, DR6 could be targeted for contrast-enhanced ultrasound imaging to detect the tumor. Thus, use of DR6-expressing epithelium in the ovary as a target may increase the sensitivity of ultrasound scanning to detect OVCA together with serum levels of DR6. Hence, it will bring a significant change in imaging paradigms and improve the specificity of ultrasound scanning. It will also make possible to determine the time between the tumor-associated elevation of DR6 in serum and the earliest detection of tumor by contrast-enhanced ultrasound scanning. This information will enable the detection of OVCA at an early stage and lead to the development of treatment modalities for patients with OVCA. In addition, current findings will also be useful in developing ovarian tumor-associated anti-DR6 therapies, which can be tested in laying hens. Taken together, information on the association of DR6 with the early detection of OVCA and the potential development of anti-DR6-based therapies will establish the foundation for clinical studies. It may thus ultimately lead to the development of an effective diagnostic test and therapies for OVCA at an early stage.

In conclusion, this study showed that the tissue expression of DR6, a potential tumor-associated neoangiogenic and immunosuppressive factor, was significantly higher in hens with early-stage OVCA than in hens with normal ovaries and increased further as the disease progressed to late stages. This information will be useful and contribute to clinical studies to determine the role of DR6 in OVCA development and progression in humans.

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# Association of Interleukin 16 With the Development of Ovarian Tumor and Tumor-Associated Neoangiogenesis in Laying Hen Model of Spontaneous Ovarian Cancer

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**Objective:** Tumor-associated neoangiogenesis (TAN) is an early event in ovarian tumor development. Interleukin 16 (IL-16) is a proangiogenic cytokine that stimulates production of neoangiogenic factors. The goal of this study was to determine the association of IL-16 with tumor development and ovarian TAN in laying hens, an animal model of spontaneous ovarian cancer (OVCA).

**Methods:** Sera and ovarian tissues from 3-year-old laying hens were collected and processed for histopathologic, immunoassay, immunohistochemistry, immunoblotting, and molecular biological studies to determine the tissue expression and serum levels of IL-16. Samples were divided into 3 groups based on the diagnosis of the histopathologic ovarian tissue examination, namely, normal (healthy control, n = 81), early (n = 23 including 11 with microscopic OVCA), and late stages (n = 16) of OVCA.

**Results:** Serum levels of IL-16 were significantly higher in hens with microscopic, early, and late stages of OVCA than normal hens ( $P < 0.0001$ ). The frequencies of IL-16<sup>+</sup> cells in tumor-bearing ovaries were significantly higher than normal hens ( $P < 0.05$ ). The expression of IL-16 protein and mRNA were stronger in tumor-bearing ovaries than normal ovaries. In addition to ovarian stroma, IL-16 was also expressed by the epithelial cells of the tumor in OVCA hens. Differences in serum levels and ovarian IL-16 expression were not significant among different histological subtypes of OVCA including serous, endometrioid, and mucinous. Similar to the serum levels and ovarian expression of IL-16, the densities of neoangiogenic microvessels were significantly higher in hens with tumor-bearing ovaries than normal hens.

**Conclusions:** The results of the study suggest that changes in serum levels of IL-16 are associated with tumor development and TAN. Thus, serum IL-16 levels may be an indicator of ovarian TAN at the early stage of OVCA.

**Key Words:** Interleukin 16, Ovarian tumor, Tumor-associated neoangiogenesis, Ovarian cancer

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Non-specificity of symptoms at an early stage and the lack of an effective early detection test make ovarian cancer (OVCA) a fatal malignancy in women.<sup>1</sup> Circulating levels of CA-125 alone or in combination with transvaginal ultrasound scan did not substantially improve the diagnosis of early-stage OVCA. Thus, an effective early detection test for OVCA remains to be established. Similar to cancers of other organs, tumor-associated neoangiogenesis (TAN) is also an early event during ovarian tumor development, and it plays critical roles in tumor growth and progression.<sup>2,3</sup> Thus, ovarian TAN vessels have the potential to be a target marker for early detection of OVCA. Doppler ultrasound scanning (DUS), a favored method for the detection of ovarian vascularity, cannot detect early ovarian TAN-related microvessels. Although vascular endothelial growth factor is a marker of TAN, it is not specific to OVCA.<sup>4</sup> Thus, for effective early detection of OVCA, either the current detection limit of DUS needs to be improved, or an effective serum marker of TAN needs to be explored.

Cytokines are reported to play putative roles in OVCA progression by enhancing tumor growth or metastasis or modulating the immune system.<sup>5</sup> Interleukin 16 (IL-16) is a cytokine involved in multiple immunopathobiological processes including chemotaxis of immune cells,<sup>6</sup> initiation of inflammatory responses,<sup>7</sup> and production of proangiogenic cytokines.<sup>8</sup> Interleukin 16 is produced mainly by members of the immune system.<sup>6</sup> Recently, IL-16 levels have been reported to be altered in association with several advanced-stage solid tumors (breast, gastrointestinal, uterine renal/bladder, and lung cancers),<sup>9</sup> hematologic malignancies,<sup>10</sup> and nonmalignant diseases including lupus and rheumatoid arthritis. An association between the serum levels of IL-16 and the early ovarian TAN has not yet been reported. If such an association exists, IL-16 may be used as a soluble marker to aid in vivo DUS detection of early ovarian TAN. Because it is difficult to identify patients at the early-stage OVCA, access to the patient's specimen remains a significant barrier to study existence of such an association.

Laying hens are the only widely available and easily accessible animals that develop OVCA spontaneously with a high incidence rate.<sup>11</sup> The histopathology and expression of several markers of OVCA in hens are similar to humans.<sup>11,12</sup> As in humans, IL-16 in chicken is synthesized as a precursor protein (pro-IL-16), with 49% to 52% identity to mammalian homologs.<sup>13</sup> Our goal was to determine whether the serum levels of IL-16 is associated with early-stage ovarian TAN in hens. We tested the hypothesis that serum levels of IL-16 are increased during ovarian tumor development and associated with early ovarian TAN.

## MATERIALS AND METHODS

### Animals

Commercial strains of 3-year-old white leghorn laying hens (*Gallus domesticus*) were maintained under standard poultry husbandry practices. The incidence of OVCA in hens of this age group is approximately 15% to 20% and is associated with low or complete cessation of egg laying.<sup>14</sup> Hens

(n = 120) were selected on the basis of their egg-laying rates (normal or low) and transvaginal ultrasound as reported previously.<sup>14</sup> All experimental procedures were performed according to the Institutional Animal Care and Use Committee-approved protocol.

### Tissue Collection and Processing

#### Serum Samples

Blood was obtained from brachial veins of all hens before they were killed, and the blood was centrifuged (1000g, 20 minutes), and serum samples were stored at  $-80^{\circ}\text{C}$ .

#### Gross Ovarian Morphology and Histopathology

Ovarian pathology and tumor staging were performed by gross and histological examination as reported previously.<sup>11</sup> Each normal or tumor-bearing ovary was divided into 4 portions for protein extraction, total RNA collection, and paraffin and frozen embedding for routine histology and immunohistochemical studies as reported previously.<sup>5</sup> Normal ovarian surface epithelial cells or epithelial cells of the tumor in OVCA hens were collected similarly as reported earlier.<sup>15</sup> Samples were classified into 3 groups including normal-, early-, and late-stage OVCA based on the diagnosis of the histopathologic ovarian tissue examination as reported previously.<sup>11</sup>

#### Preparation of Ovarian Specimen for Biochemical Analysis

Snap-frozen ovarian tissues as well as normal ovarian surface epithelial cells and epithelial cells of the tumor in OVCA hens were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) as reported previously<sup>16</sup> and centrifuged, and supernatant was collected; protein content of the extract was measured and stored at  $-80^{\circ}\text{C}$ .

#### Immunoassay

Serum levels of IL-16 were determined by Chicken IL-16 Vetset ELISA Kit (Kingfisher Biotech, St Paul, MN) as per the manufacturer's instructions. Briefly, chicken IL-16 standards or serum samples were added to the wells precoated with anti-chicken IL-16 antibodies followed by the incubation with detection antibody and streptavidin-horseradish peroxidase. Immunoreactions were developed by TMB substrate and stopped by 0.18 M sulfuric acid. The absorbance for each well was recorded by reading the plates at 450 nm in a plate reader (Thermomax; Molecular Devices, Sunnyvale, CA). A standard curve was generated by plotting the optical density values of the standards against their concentrations. Serum IL-16 levels were determined with reference to the standard curve as per manufacturer's instruction using a software program (Softmax Pro, version 1.2.0, software; Molecular Devices). All standards and serum samples were run in duplicate.

#### Immunohistochemistry

Immunohistochemical localizations of IL-16<sup>+</sup> cells using anti-IL-16 (Kingfisher Biotech) and ovarian TAN vessels



using anti-smooth muscle actin (SMA) (Invitrogen, Carlsbad, CA) and anti- $\alpha_v\beta_3$  integrins (CD51/CD61, clone 23C6; BioLegend, San Diego, CA) antibodies in normal or tumor-bearing ovaries ( $n = 15$  hens each for normal, early [including microscopic] and late stages) were performed as reported previously.<sup>3</sup> The number of hens for each group for immunohistochemical studies was determined based on the power analysis to achieve significant differences in the frequencies of immunopositive cells or microvessels among the 3 pathological groups. These hens were selected from each group randomly based on their reactivity in immunoassay. Double label immunostaining was performed to determine whether IL-16 is expressed by normal ovarian surface epithelium or epithelium of the tumor in OVCA hens. Briefly, frozen sections from normal or tumor-bearing ovaries were immunostained for cytokeratin (a marker of epithelial cell) using anti-cytokeratin 7 antibodies (this antibody was developed against OTN 11 ovarian carcinoma cell lines; Abcam Inc, Cambridge, MA) in the first step and anti-IL-16 antibodies in the second step. Cytokeratin-positive cells were visualized with DAB, and IL-16<sup>+</sup> cells were detected by DAB with nickel chloride as reported earlier.<sup>17</sup> Sections were then counterstained with hematoxylin, dehydrated, and covered. In control staining, first antibody was replaced with normal mouse immunoglobulin G, and no immunopositive cell was found on the control slides.

### Counting of Immunopositive Cells or Microvessels

The densities of IL-16<sup>+</sup> cells, anti-SMA<sup>+</sup>, and anti- $\alpha_v\beta_3$  integrin<sup>+</sup> microvessels were counted from the normal ovarian or tumor stroma of hens using a light microscope attached to a digital imaging software (MicroSuite version 5; Olympus Corporation, Tokyo, Japan). Three sections per ovary were selected. In each section, 5 regions containing a high population of immunopositive microvessels or 5 random areas for IL-16<sup>+</sup> cells were used. Frequencies of IL-16<sup>+</sup> cells or microvessels in 20,000- $\mu\text{m}^2$  area were counted at a  $\times 40$  objective and  $\times 10$  ocular magnification as reported previously.<sup>3,18</sup> The mean of these counts was considered as the number of immunopositive microvessels or IL-16<sup>+</sup> cells in a 20,000- $\mu\text{m}^2$  area of a section. The mean of 3 sections was considered as the mean of immunopositive vessels or IL-16<sup>+</sup> cells in normal or tumor-bearing ovaries.<sup>3</sup>

### One-Dimensional Western Blot

Serum samples for Western blotting were selected randomly to represent normal ( $n = 5$ ), early including microscopic ( $n = 12$ ), and late stages ( $n = 12$ ) of OVCA based on their immunoreactivity for IL-16 in immunoassay (enzyme-linked immunosorbent assay). Serum samples were filtered by acetonitrile and chloroform-methanol precipitation and tested by immunoblotting to confirm immunoreactions observed in immunoassay as reported earlier.<sup>16</sup> Similarly, ovarian expression of IL-16 was confirmed by using whole ovarian homogenates and normal ovarian surface epithelial cells or epithelial cells of the tumor in OVCA hens. Immunoreactions on the membrane were visualized as a chemiluminescence product (Super Dura West substrate; Pierce/Thermo Fisher, Rockford,

IL), and the image was captured using a Chemidoc CRS (BioRad, Hercules, CA). Chicken recombinant IL-16 protein was used as standard in immunoblotting.

### Reverse Transcription-Polymerase Chain Reaction

Interleukin 16 mRNA expression was assessed by semi-quantitative reverse transcription-polymerase chain reaction (PCR) as reported previously.<sup>19</sup> For reverse transcription-PCR analyses, serum and tissue samples from 5 normal, 12 early (including microscopic, 4 from each of the serous, endometrioid, and mucinous), and 12 late stages (4 from each of the serous, endometrioid, and mucinous) of OVCA hens were selected based on their reactivity in immunoassay, immunoblotting, and immunohistochemistry. Hen-specific IL-16 primers were designed by Oligoperfect Designer software (Invitrogen) using the IL-16 sequence from the NCBI (GeneBank NM\_204352.3). The forward primer was 5-TCTCTGCTTCCCTGAA-GA, and the reverse primer was 5-GTCATTGGGAAACACCT-TG located between exons 4 and 6.  $\beta$ -Actin was used as the endogenous control with a forward primer of TGCGTGACATCAAGGAGAAG and a reverse primer of ATGCCAGGGTACATTGTGGT. The expected base pair size for the IL-16 amplicon was 199 base pairs and for  $\beta$ -actin was 300 base pairs. Polymerase chain reaction amplicons were visualized in a 3% agarose gel (Pierce/Thermo Fisher) in Tris-acetate-EDTA buffer and stained with ethidium bromide. The image was captured using a ChemiDoc XRS system (BioRad).

### Statistical Analysis

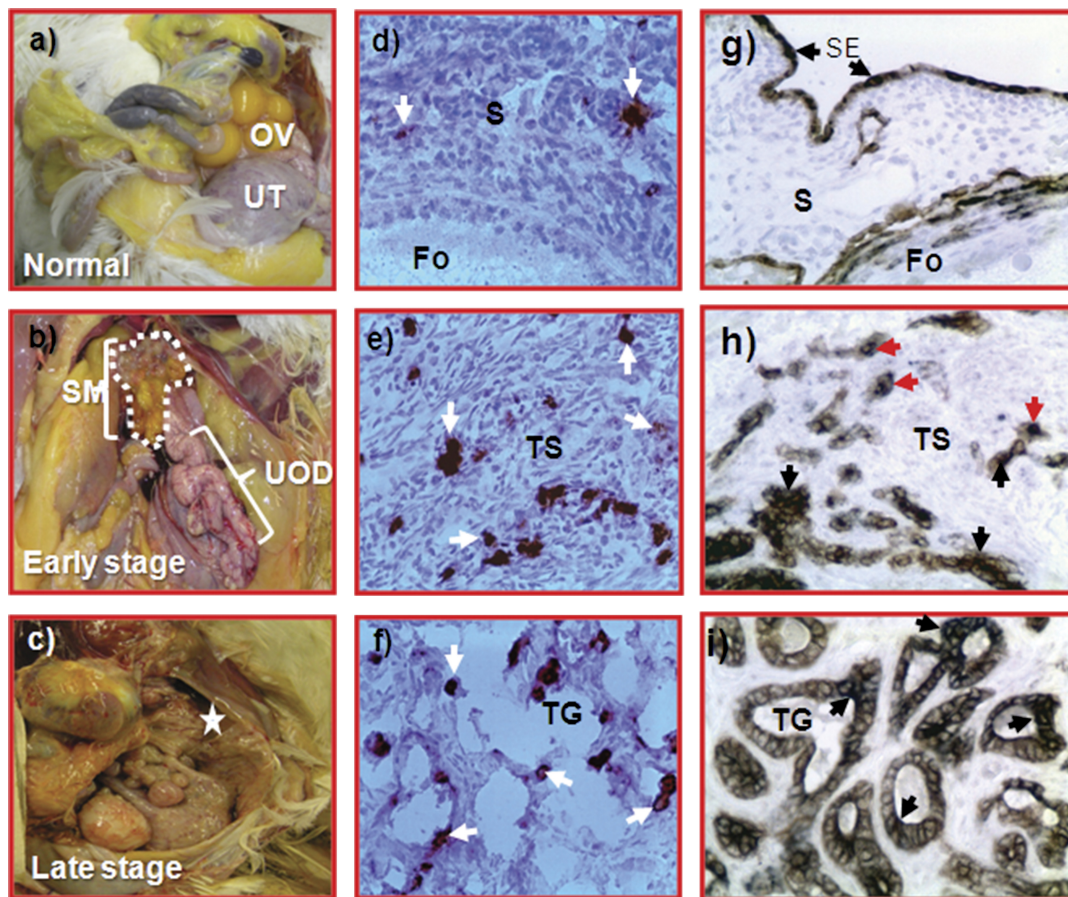
The differences in the frequencies of IL-16<sup>+</sup> cells and immunopositive microvessels among normal ovaries, tumor-bearing ovaries in hens with early-stage and late-stage OVCA were assessed by analysis of variance, F tests, and the alternative nonparametric Kruskal-Wallis tests. Then pairwise comparisons between the groups (normal-, early-, and late-stage OVCA) by 2-sample *t* tests and alternative Mann-Whitney tests were performed. All reported *P* values are 2-sided, and *P* < 0.05 was considered significant. Statistical analyses were performed with SPSS (PASW) version 18 software (IBM Inc, Armonk, NY).

## RESULTS

### Ovarian Morphologic and Histological Features

#### Gross Morphology

Hens with a fully functional ovary contained 5 to 6 developing preovulatory follicles (Fig. 1A). Only 1 or 2 such follicles were present in the ovaries of low-laying healthy hens. In normal hens that stopped egg laying, ovaries and oviducts were regressed without any detectable abnormality. Solid tissue masses either limited to a small part or whole ovary and with or without ascites were observed in 12 hens and classified as hens with early-stage OVCA (Fig. 1B). In 16 hens, tumor metastasized to abdominal organs with moderate



**FIGURE 1.** Gross morphology and immunolocalization of IL-16<sup>+</sup> cells in normal ovaries and ovaries with tumor in laying hens. A–C, Gross morphology of normal ovary or ovaries with tumors in hens. A, A normally laying hen ovary contains a hierarchy of multiple large preovulatory follicles with active oviduct. B, Ovarian cancer at an early stage in a laying hen. Tumor mass is limited to the ovary (shown in a dotted line), and the oviduct is atrophied. C, Late-stage OVCA in a laying hen. Tumor is metastasized to distant organs with accompanied profuse ascites (\*). D–F, Interleukin 16–expressing cells in the stroma of a normal ovary or ovaries with tumors in laying hens. Frozen sections from ovaries shown in A to C were immunostained for IL-16–expressing cells. Cells expressing IL-16 are round or irregularly shaped and seen in the normal ovarian stroma as well as in the vicinity of tumors. D, Very few positive cells are seen in the stroma of normal ovary. E and F, Interleukin 16 expression in early (E) and late (F) stages of OVCA in laying hens. A number of epithelial cells (not all) of the tumor in the ovaries were also positive for IL-16 expression (E–F). Compared with normal ovary (D), more IL-16<sup>+</sup> cells are seen in the stroma of the ovaries with tumor (E–F) in OVCA hens. G–I, Expression of IL-16 by ovarian epithelium in hens with or without OVCA. Frozen sections were immunostained for cytokeratin 7 (a specific marker for epithelial cell) in the first step to confirm ovarian epithelial cells and for IL-16 in the second step using DAB and DAB and nickel chloride, respectively. Cytokeratin 7–positive epithelial cells expressing IL-16 appeared as gray-black (examples shown in black arrows) compared with light brown non-IL-16–expressing cytokeratin 7–positive cells. G, Interleukin 16 expression by normal ovarian surface epithelium. H and I, Interleukin 16 expression by the epithelial cells of the tumor in hens with early (H) and late stages (I) of OVCA. In addition to the epithelial cells, some rounded cells (with T cell–like phenotype) were stained dark green for IL-16 only (red arrows) in the stroma of the ovaries with tumor (H). S, Stroma; SE, surface epithelium, SM, solid mass, TG, tumor gland; TS, tumor stroma, UOD, upper oviduct, UT, uterus. \*Ascites. Original magnification  $\times 40$ . Arrows indicate the examples of immunopositive cells.

to profuse ascites and classified as hens with late-stage OVCA (Fig. 1C).

### Histopathology

Ovarian tumors in 28 hens that had solid mass limited to the ovaries (n = 12, early stage) or metastasized to other

organs (n = 16, late stage) were confirmed by routine histology. However, tumor-associated microscopic features were also found during histological examinations in 11 additional hens (hens with microscopic OVCA) that had no grossly detectable solid mass in the ovary and were grouped in early-stage OVCA. Thus, a total of 23 (12 + 11) hens had early and



16 had late stage of OVCA, whereas 81 hens were normal. Tumors were typed as serous ( $n = 17$ ), endometrioid ( $n = 12$ ), mucinous ( $n = 8$ ), and clear cell ( $n = 1$ ) as well as mixed ( $n = 1$ , seromucinous) as reported previously.<sup>11</sup>

## Tissue Expression and Serum Levels of IL-16

### Immunohistochemical Detection of Ovarian IL-16 Expression

Rounded (T lymphocyte-like) to irregularly shaped (macrophage-like appearances) IL-16<sup>+</sup> cells were detected in the stroma of normal or tumor-bearing ovaries and in the tumor vicinity including spaces between tumor glands (Figs. 1D–F). A number of epithelial cells (not all) in normal or tumor glands were also positive for IL-16 (Figs. 1D–F), which was confirmed by double immunostaining for IL-16 together with cytokeratin (Figs. 1G–I). The frequencies of IL-16<sup>+</sup> cells in the stroma of tumor-bearing ovaries in hens with early-stage (mean, 15.88 [SD, 3.33] cells/20,000- $\mu\text{m}^2$  area) and late-stage (19.5 [SD, 4.90] cells/20,000- $\mu\text{m}^2$  area) OVCA were significantly ( $P < 0.05$ ) higher than in the ovarian stroma of normal hens (5.24 [SD, 1.38] cells/20,000- $\mu\text{m}^2$  area). Thus, epithelial cells in normal ovaries or tumor-bearing ovaries also express IL-16.

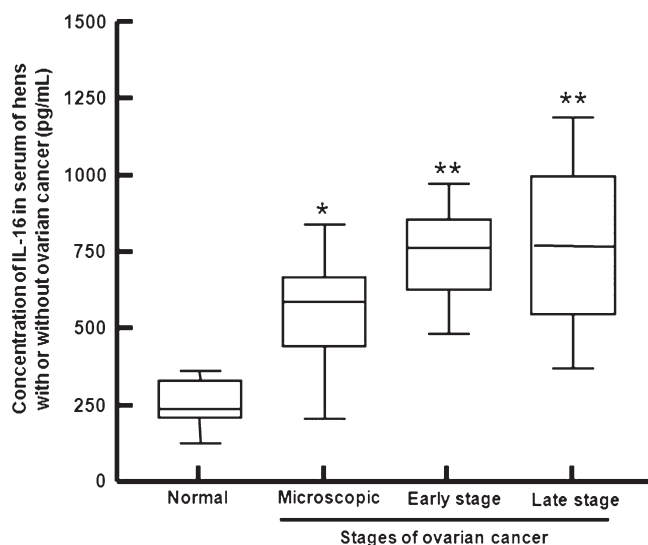
### Immunoassay for Serum IL-16 Levels

Mean concentration of serum IL-16 was 259.91 (SD, 71.90) pg/mL in normal hens ( $n = 50$ ). Compared with normal hens, the mean concentration of serum IL-16 was significantly higher ( $P < 0.0001$ ) in hens with microscopic OVCA (531.85 [SD, 193.77] pg/mL) and increased further in hens with early (739.55 [SD, 148.75] pg/mL) and late stages (767.04 [SD, 264.37] pg/mL) of OVCA (Fig. 2). Although compared with hens with microscopic OVCA, serum IL-16 levels were significantly higher in hens with early ( $P < 0.009$ ) and late stages ( $P < 0.004$ ) of OVCA, differences in serum IL-16 levels were not significant between the early- and late-stage OVCA as well as among the different histological subtypes of OVCA.

### Immunoblotting for IL-16 Protein in Serum Samples and Ovarian Tissues

Immunoreactivity of serum IL-16 detected by immunoassay was confirmed with immunoblotting using serum samples from selected hens representative of each group including normal-, early-, and late-stage OVCA. As expected, a band at approximately 15 to 18 kD was detected in recombinant IL-16 samples (data not shown), whereas a band of approximately 60 to 65 kD (equivalent to the homotetramer of recombinant IL-16 peptide) was detected in the serum from normal hens or hens with OVCA (Fig. 3A).

Expression of ovarian IL-16 was also confirmed by immunoblotting using ovarian epithelium or whole ovarian homogenates from normal hens or hens with OVCA. Interleukin 16 protein from whole ovarian homogenates in hens with OVCA showed moderate to intense immunoreactivity

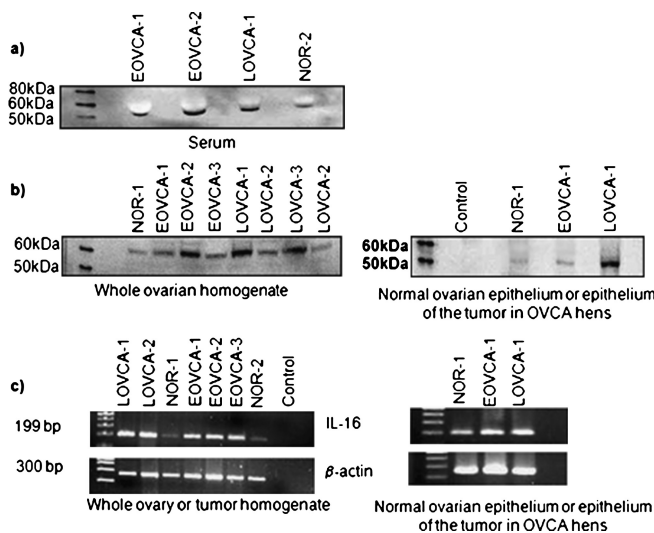


**FIGURE 2.** Interleukin 16 levels in serum of hens with or without ovarian tumors displayed as box and whiskers (expressed in pg/mL). The median, range (whiskers), and 25th to 75th percentiles (box) are shown. Serum samples from normal hens were used as experimental controls. Compared with control hens ( $n = 50$ ), serum IL-16 levels increased significantly ( $P < 0.0001$ ) in hens with microscopic ( $n = 11$ ), early ( $n = 12$ ), and late ( $n = 16$ ) stages of OVCA. \*Significant differences in the serum IL-16 levels between normal hens and hens with microscopic OVCA; \*\*same between the hens with microscopic OVCA and hens with early or late stages of OVCA.

(Fig. 3B). In contrast, either a very weak or no immunoreactive band was seen for whole ovarian homogenates from healthy hens. Similarly, stronger IL-16 immunoreactivity was observed for the epithelial cells of the tumor in OVCA hens than that of normal ovarian surface epithelial cells (Fig. 3B). These results confirm the immunohistochemical observation that ovarian surface epithelial cells in normal hens and epithelial cells of the tumor in OVCA hens may be a source of IL-16 proteins in hens.

### Expression of IL-16 Messenger RNA

Interleukin 16 messenger RNA (mRNA) expression confirmed the observed variations in serum IL-16 levels and ovarian IL-16 expression among normal and OVCA hens. Hens with ovarian tumors showed strong amplification signal for IL-16 (Fig. 3C) and differences were not observed in IL-16 mRNA expression among different histological subtypes of OVCA. In contrast, IL-16 mRNA expression was weak for whole ovarian homogenates from normal hens. Similar patterns were also observed for surface epithelial cells of the normal ovaries or epithelial cells of the tumor in OVCA hens. Overall, compared with normal hens, strong amplification of IL-16 mRNA was observed in OVCA hens as observed for immunoreactivities in immunoassay and immunoblotting.

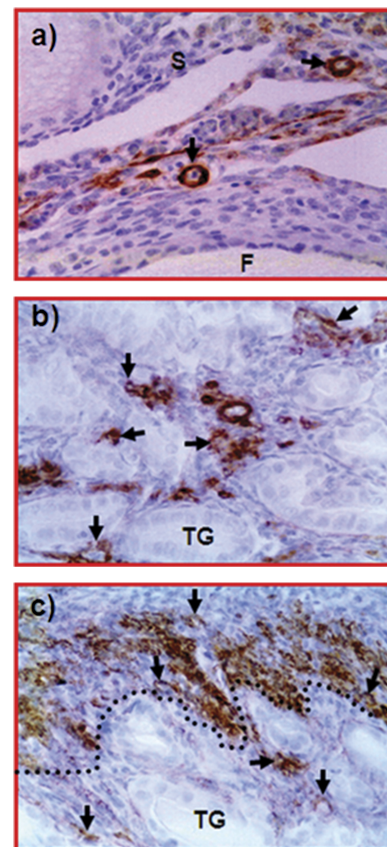


**FIGURE 3.** Examples of immunoreactive IL-16 protein (A–B) or mRNA expression (C) in the sera and homogenates of whole ovarian or ovarian epithelium from normal hens (NOR) or epithelium of the tumor in hens with early (EOVCA) and late (LOVCA) stages of OVCA by 1-dimensional Western blotting (1D-WB) and semiquantitative PCR, respectively. 1D-WB (A–B): Immunoreactive IL-16 proteins of 55- to 60-kd molecular weight were detected in sera and homogenates of ovaries from normal hens or hens with ovarian tumors as well as epithelium of the tumor in OVCA hens. Compared with sera and ovaries from normal hens, relatively stronger immunoreactive bands for IL-16 proteins were observed in hens with OVCA. No immunoreactive band was detected in the negative control in which protein sample was omitted. Reverse transcription–PCR (C): mRNA expression was detected in the extract from whole ovarian homogenate or epithelium of the tumor in OVCA hens. Compared with the weak expression by normal ovaries, strong expression of IL-16 mRNA was observed in homogenates of ovarian tumors and epithelium of the tumor in hens with early- and late-stage OVCA. No IL-16 mRNA expression was detected in negative control in which mRNA sample was omitted.

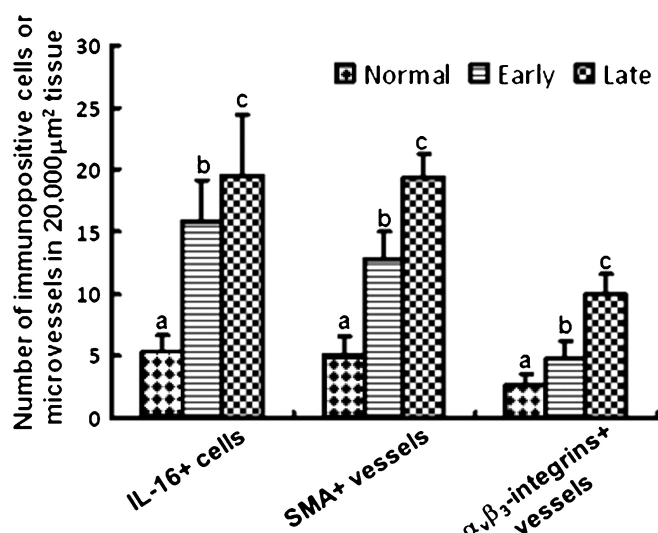
### Expression of Angiogenic Markers

Immunopositive microvessels (anti-SMA<sup>+</sup> and anti- $\alpha_v\beta_3$  integrin<sup>+</sup>) were detected in both normal ovaries ( $n = 15$ ) and ovarian tumors ( $n = 15$  hens each for early- and late-stage OVCA). In normal hens, the wall of immunopositive ovarian vessels, in most cases, was thick, complete, and continuous with intense staining. A few microvessels were also seen in the theca layers of the follicles of normal hens, indicating active sites of physiological angiogenesis associated with follicular development (Fig. 4A). In contrast, most of the immunopositive microvessels in tumor-bearing ovaries were leaky, discontinuous, or incomplete with thin vessel walls. In OVCA hens, more immunopositive microvessels were

seen in tumor vicinity and in the stroma adjacent to the tumors (Figs. 4B, C). The frequencies of immunopositive microvessels were significantly ( $P < 0.0001$ ) higher (12.77 [SD, 2.19] anti-SMA and 6.77 [SD, 3.00] anti- $\alpha_v\beta_3$  integrins<sup>+</sup> microvessels in 20,000- $\mu\text{m}^2$  area of tissues) in early-stage OVCA hens than normal hens (5.06 [SD, 1.46] anti-SMA and 2.7 [SD, 0.84] anti- $\alpha_v\beta_3$  integrins<sup>+</sup> microvessels in 20,000- $\mu\text{m}^2$  area of tissues) (Fig. 5). Their frequencies increased further ( $P < 0.0001$ ) in hens with late-stage OVCA (19.39 [SD, 1.95] anti-SMA and 9.97 [SD, 1.65] anti- $\alpha_v\beta_3$  integrins<sup>+</sup> microvessels in 20,000- $\mu\text{m}^2$  area of tissues).



**FIGURE 4.** Immunolocalization of ovarian microvessels in normal ovary or ovaries with tumor in laying hens. A, Ovarian section from a normal hen. Few immunopositive microvessels are seen in the ovarian stroma. B, Section of a hen's ovary with early-stage OVCA. Compared with the normal ovary, more immature immunopositive microvessels are present in the spaces between tumor glands. C, Section of an ovary with tumor from a hen with late-stage OVCA. Compared with the normal ovary and early-stage OVCA, many microvessels are seen in between tumor glands as well as in the stroma adjacent to the tumor, indicating that tumor angiogenesis precedes tumor progression (separated by an imaginary dotted line). Arrows indicate examples of immunopositive microvessels. F, Follicle; S, stroma; TG, tumor gland.



**FIGURE 5.** Changes in the ovarian IL-16 expression are associated with the increase in the frequencies of ovarian tumor-associated microvessels. The frequencies of immunopositive IL-16 cells or microvessels are expressed as the mean (SD) in 20,000- $\mu\text{m}^2$  area. As compared with normal hens ( $n = 15$  hens), the frequencies of the IL-16<sup>+</sup> cells as well as angiogenic microvessels were significantly increased in hens with OVCA ( $n = 15$  hens each for early including microscopic and late stages of OVCA). The increase in the frequency of ovarian IL-16-expressing cells is associated with the increase in the frequencies of SMA and  $\alpha_v\beta_3$  integrin-expressing microvessels. Bars with different letters indicate significant differences ( $P < 0.001$ ) among different pathological categories in IL-16<sup>+</sup> cell, SMA<sup>+</sup>, or  $\alpha_v\beta_3$  integrins expressing microvessel groups.

Similar to serum IL-16 levels, the densities of immunopositive microvessels were also increased with the development of OVCA in hens.

## DISCUSSION

This is the first report that serum levels and tissue expression of IL-16 increase significantly in association with the development and progression of ovarian TAN in laying hens, a preclinical animal model of spontaneous OVCA. These results suggest that serum IL-16 levels may be a potential indicator of early-stage ovarian TAN and may be useful in combination with conventional markers including vascular endothelial growth factor to detect ovarian TAN.

Precise molecular mechanism of the initiation and regulation of ovarian TAN is not well understood. Involvement of several cytokines and growth factors in the development of TAN has been reported.<sup>5</sup> Interleukin 16 was reported to be involved<sup>9</sup> in lymphoid and ovarian malignancies,<sup>5,10</sup> but none of these reports examined its association with early stages of malignancies. As in humans,<sup>5</sup> the present study showed that IL-16 in hen serum and ovarian tissues also exists

in the active homotetramer form. In this study, compared with normal hens, serum levels and tissue expression of IL-16 increased significantly in OVCA hens including microscopic OVCA. The reason(s) for this increase in IL-16 levels in OVCA hens is not known. However, this study suggests that tumor-associated increase in IL-16 level can be detected in serum even before the tumor becomes detectable by TUVS (microscopic OVCA). Furthermore, this increase was correlated with the increased density of TAN-related microvessels. Thus, increased serum IL-16 levels, as observed in hens with early-stage OVCA, may represent an effective serum indicator of early-stage ovarian TAN.

The sources of serum IL-16 are not yet fully known, and published reports are very limited.<sup>20,21</sup> In addition to the immune cells, IL-16 has been reported to be secreted by bronchial epithelium in humans.<sup>20,21</sup> In this study, normal ovarian surface epithelial cells and epithelial cells of the tumor in OVCA hens were positive for IL-16 expression, suggesting that these tissues may also be a source of serum IL-16. Double immunohistochemical labeling showed the expression of IL-16 by the surface epithelial cells of the normal ovaries and epithelial cells of the tumor in OVCA hens, which was further supported by IL-16 protein and mRNA expression. These results are consistent with the previous report that bronchial and tracheal epithelial cells also secrete IL-16.<sup>21</sup> Thus, normal ovarian surface epithelial cells and epithelial cells of the tumor in OVCA hens were also a source of IL-16, and secretion of IL-16 by the epithelial cells of the tumor may be one of the reasons for elevated serum IL-16 levels in OVCA hens including those with microscopic OVCA.

Although the physiological significance of epithelial cell-derived IL-16 in the pathogenesis of OVCA is not clear, it is possible that IL-16 may be involved in the development and progression of OVCA. First, the positive correlation between the increase in IL-16 levels and the frequency of TAN vessels in OVCA hens suggests a proangiogenic role of IL-16 in the progression of ovarian TAN. A proangiogenic role of IL-16 has also been reported in humans.<sup>8</sup> Second, increased expression of IL-16 by epithelial cells of the tumor in OVCA hens and increased levels of serum IL-16 in hens with microscopic OVCA even before the tumor becomes detectable suggest that serum IL-16 levels may also be an indicator of malignant ovarian transformation. Thus, it is possible that IL-16 secreted by the epithelial cells of the tumor in OVCA hens may contribute to ovarian TAN required for tumor growth and may be an effective indicator of ovarian tumorigenesis.

Because of the difficulty of detecting OVCA at an early stage, access to patient specimens to study and develop an early detection test is difficult and time consuming. Because of the similarities between the spontaneous OVCA in humans and hens including histological subtypes, molecular pathways, risk factors (eg, incessant ovulation), and expression of several molecular markers of OVCA, there is a high probability that the results obtained from the OVCA studies in laying hens may be translated to clinics. Moreover, one of the most important advantages relative to the translationalities of the current findings is that OVCA development in



hens can be monitored by contrast-enhanced ultrasound imaging using equipment and mechanical setting similarly used in clinics.<sup>3,14</sup> Thus, there will be least variation on imaging parameters between hens and humans because of the equipment. With the advancement in in vivo imaging technology, ligands that bind to the epithelial cells of the tumor in OVCA hens are being developed to use in conjunction with microbubbles for contrast-enhanced ultrasound scanning to facilitate early OVCA detection.<sup>18</sup> As the epithelial cells of the tumor in OVCA hens express IL-16, it may be used as a target for contrast-enhanced ultrasound imaging and will increase the sensitivity of ultrasound scanning detection of OVCA together with serum levels of IL-16. Thus, it will bring a significant change in imaging paradigms and improve the specificity of ultrasound scanning. It will also be possible to determine the time between the tumor-associated elevation of IL-16 in serum and the earliest detection of tumor by contrast-enhanced ultrasound scanning. This information will enable to detect OVCA at an early stage and lead to the development treatment modalities for OVCA patients. In addition, current findings will also be useful in developing ovarian tumor-associated anti-IL-16 therapies and can be tested in laying hens. Taken together, information on the association of serum and tissue expression of IL-16 with the early detection of OVCA and the potential development of anti-IL-16-based therapies in laying hens will establish the foundation for clinical studies that may ultimately lead to the development of an effective diagnostic test and therapies for OVCA at an early stage.

One of the limitations of the present study is that it did not examine the serum levels and tissue expression of IL-16 in ovarian benign tumors. Thus, exclusion of ovarian benign tumors from the study may limit the translational significance of the findings that serum IL-16 levels may be used as a biomarker for early detection of OVCA. However, serum levels of IL-16 were reported to be significantly higher in patients with prostate cancer than patients with benign prostatic hyperplasia.<sup>22</sup> Therefore, to evaluate the effectiveness of serum IL-16 as a biomarker for early OVCA detection or as a target for imaging of ovarian tumors in patients with early-stage OVCA, present observations need to extend to the investigations on benign ovarian tumors and other benign conditions associated with de novo angiogenesis or IL-16 elevations.

The laying hen is a suitable model to study the role of IL-16 in the pathogenesis of OVCA. With the emergence of newer in vivo imaging technologies, effective targets in the tumor in OVCA hens are being identified to be detected by targeted contrast-enhanced ultrasound imaging to facilitate early OVCA detection. As the epithelial cells of the tumor in OVCA hen express IL-16, these cells may be used as a target for such molecular targeted ultrasound imaging. Thus, serum IL-16 levels together with ultrasound imaging of ovarian tumor-associated IL-16 expression may constitute an effective test for early OVCA. Laying hens are also a suitable model for the development of ultrasound contrast agents for OVCA detection.<sup>18</sup> Finally, the laying hen may also be used to test the efficacy of anti-IL-16 therapies for effective prevention of ovarian TAN.

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## Expression of Leukocyte Inhibitory Immunoglobulin-like Transcript 3 Receptors by Ovarian Tumors in Laying Hen Model of Spontaneous Ovarian Cancer<sup>1</sup>

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### Abstract

Attempts to enhance a patient's immune response and ameliorate the poor prognosis of ovarian cancer (OVCA) have largely been unsuccessful owing to the suppressive tumor microenvironment. Leukocyte immunoglobulin-like transcript 3 (ILT3) inhibitory receptors have been implicated in immunosuppression in several malignancies. The expression and role of ILT3 in the progression of ovarian tumors are unknown. This study examined the expression and association of ILT3 in ovarian tumors in laying hens, a spontaneous preclinical model of human OVCA. White Leghorn laying hens were selected by transvaginal ultrasound scanning. Serum and normal ovaries or ovarian tumors were collected. The presence of tumors and the expression of ILT3 were examined by routine histology, immunohistochemistry, Western blot analysis, and reverse transcription–polymerase chain reaction. In addition to stromal immune cell-like cells, the epithelium of the ovarian tumors also expressed ILT3 with significantly high intensity than normal ovaries. Among different subtypes of ovarian carcinomas, serous OVCA showed the highest ILT3 staining intensity, whereas endometrioid OVCA had the lowest intensity. Similar to humans, an immunoreactive protein band of approximately 55 kDa for ILT3 was detected in the ovarian tumors in hens. The patterns of ILT3 protein and messenger RNA expression by ovarian tumors in different subtypes and stages were similar to those of immunohistochemical staining. The results of this study suggest that laying hens may be useful to generate information on ILT3-associated immunosuppression in OVCA. This animal model also offers the opportunity to develop and test anti-ILT3 immunotherapy to enhance antitumor immunity against OVCA in humans.

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### Introduction

Despite the remarkable improvements in cytoreductive surgeries and chemotherapeutics, ovarian cancer (OVCA) remains one of the most lethal gynecologic malignancies of women with a high death rate [1]. Owing to the lack of an effective early detection test, OVCA in most cases is detected at late stages, and its high recurrence rate (80%–90%) contributes to poor prognosis [2,3]. There is an emerging recognition that tumor growth, in general, elicits specific immune responses mediated by cell-mediated immunity [4]. As a result, immunotherapies against several cancers are being developed [4–6]. Although recent

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advances in immunotherapy have been shown to improve the overall survival ability of patients with hematologic tumors and melanoma [7], most immunotherapeutic trials have failed to demonstrate success in clinical responses [6,8]. Thus, development of new strategies to promote immune responses against malignancies is critical in overcoming the limited efficacy of conventional therapies. Despite the presentation of antigens by ovarian malignant cells, which should induce immune-mediated rejection, spontaneous rejection of an established tumor is rare [9]. This lack of immune response is not only because of the ignorance of the immune system but also because of the tumor-induced immune suppression that protects the tumor from eradication [9]. Therefore, a better understanding of the mechanisms of tumor-induced immunosuppression will enhance our ability to prevent ovarian tumor progression and to design antitumor interventional strategies.

Numerous studies on cancers of several organs have reported several mechanisms of tumor-induced immune suppression including induction of regulatory T cells [10], expression of immunosuppressive factors (transforming growth factor  $\beta$ , interleukin 10, and chemokine ligand 22) [10–12], down-regulation of intracellular adhesion molecules [13], and induction of peripheral tolerance [4,14,15]. In contrast, studies on the mechanism of immune suppression in ovarian malignancy are very limited. OVCA differs from other malignancies in its specific dissemination pattern [9]. The tumor typically spreads in a diffuse intra-abdominal fashion rather than through systemic circulation. Thus, antitumor immune response at the tumor environment plays a critical role to ovarian tumor metastasis. Immunosuppressive regulatory T cells [10], transforming growth factor  $\beta$  [11,12], tolerance-inducing plasmacytoid dendritic cells [16], B7-H4+ macrophages [17], and interleukin 10 [18] have been reported to be present in the tumor microenvironment. However, how these immunosuppressive factors and agents are recruited into the tumor environment is not known. Emerging studies suggest that induction of inhibitory receptor immunoglobulin (Ig)-like transcript 3 (ILT3) expression is one of the mechanisms contributing to the tumor-induced immune suppression in several malignancies [19].

ILT3 is a member of leukocyte Ig-like receptors family with inhibitory functions and exists in both membrane and soluble forms [20]. Both forms of ILT3s have been suggested to inhibit T-cell proliferation, CD4<sup>+</sup> T-cell anergy, suppressing the differentiation of interferon  $\gamma$ -producing CD8<sup>+</sup> cytotoxic lymphocytes. In addition, membrane and soluble ILT3 were also reported to stimulate the differentiation of regulatory T cells in various cancer patients [4,5,10]. All these findings suggest that ILT3 may be involved in the immunosuppression against tumor antigens and prevention or blocking of ILT3 expression may enhance a patient's immune responses to malignancies. The expression of ILT3 in OVCA patients has not yet been reported. Difficulties in identifying and access to patients at the early stage of OVCA hinder the ability to study the involvement of ILT3 in OVCA progression and develop interventional strategies for its prevention. Rodents do not develop OVCA spontaneously, and the histopathologies of induced OVCA in rodents do not resemble the spontaneous OVCA in humans [21]. Recently, we have shown that laying hens are the only widely available animals that develop OVCA spontaneously with a high incidence rate and histopathologies remarkably similar to human OVCA [22]. The expression of leukocyte Ig-like receptors has been reported in chicken, which are shown to be orthologous to those of mammals including humans [23,24]. Thus, the goal of this study was to examine whether ILT3 is expressed in ovarian tumors in the laying hen model of

spontaneous OVCA and, if so, whether ILT3 expression is associated with the progression of ovarian tumors in hens.

## Materials and Methods

### Animals

Commercial strains of approximately 3-year-old white Leghorn laying hens (*Gallus domesticus*) were selected from a flock of layers maintained under standard poultry husbandry practices. The incidence of OVCA in hens of this age group is approximately 15% to 20% and is associated with low or complete cessation of egg laying [25]. Hens ( $n = 148$ ) were selected on the basis of their egg-laying rates (normal, low, or ceased egg laying) and transvaginal ultrasound scanning as reported previously [25]. All experimental procedures were performed according to the institutional animal care and use committee approved protocol.

### Tissue Collection and Processing

**Serum samples.** Blood was obtained from brachial veins of all hens before euthanasia, centrifuged (1000g for 20 minutes), and serum samples were stored at  $-80^{\circ}\text{C}$  until further use.

**Gross ovarian morphology and histopathology.** Ovarian pathology and tumor staging were performed by gross and histologic examination as reported previously [22]. Each normal ovary or ovary with tumor was divided into four portions for protein extraction, total RNA collection, paraffin and frozen embedding for routine histology, and immunohistochemical studies as reported previously [26]. Ovarian surface epithelial (OSE) cells from normal or ovaries with tumor were collected similarly as reported earlier [27]. All collected samples were grouped into three groups including normal-, early-, and late-stage OVCA based on the diagnosis of the histopathologic ovarian tissue examination as reported previously [22].

### Preparation of Ovarian Specimen for Biochemical Analysis

Snap-frozen normal ovaries and ovaries with tumor as well as OSE from normal ovaries and ovaries with tumor were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) as reported previously [28] and centrifuged, the supernatant was collected, and the protein content of the extract was measured and stored at  $-80^{\circ}\text{C}$  until further use.

### Histopathologic Examination and Immunohistochemistry

Paraffin or frozen sections from each ovary with tumor or ovaries that appeared normal without any grossly detectable tumor were stained with hematoxylin and eosin and observed under a light microscope. Presence or absence of tumors in the section and their histologic types were determined as reported earlier [22]. Immunohistochemical detection of ILT3 expression was performed using goat anti-ILT3 (R&D Systems, Inc, Minneapolis, MN) as primary antibodies ( $n = 15$  hens each, for normal, early, and late stages as reported previously) [26]. The number of hens for each group was determined based on statistical power analysis to achieve significant differences in the intensities of ILT3 immunostaining among the hens of normal or OVCA groups. These hens were selected from each group randomly. Briefly, sections were deparaffinized, and antigens on the sections were unmasked by heating the sections with an antigen-unmasking solution (Vector Laboratories, Burlingame, CA) for 20 minutes in a microwave oven. Endogenous peroxidase in the



sections was inactivated, and nonspecific staining was blocked by incubating with 0.3% hydrogen peroxide in methanol for 30 minutes followed by 1% (vol/vol) normal horse serum for 15 minutes, respectively. Sections were then incubated overnight with primary antibodies (1:100 dilution) followed by 1 hour of incubation with anti-goat IgG-HRP secondary antibodies (R&D Systems). Immunoprecipitates on the sections were visualized by incubation with a mixture of diaminobenzidine and hydrogen peroxide in diaminobenzidine buffer (Vector Laboratories). Sections were then counterstained with hematoxylin, dehydrated, and covered. Control staining was carried out simultaneously in which the first antibodies were omitted and normal goat serum was used. No staining was found in these control slides.

Sections were then examined under a light microscope attached to digital imaging software (MicroSuite version 5; Olympus Corporation, Tokyo, Japan). Three sections per ovary and five regions of interest (20,000  $\mu\text{m}^2$ /region at  $\times 40$  objective and  $\times 10$  ocular magnification) per section were randomly selected. Using the software, the intensity of the ILT3 immunostaining in each region was measured and recorded as pixel values in 20,000  $\mu\text{m}^2$  of the section. The mean of pixel values of these five regions in a section was considered as the intensity of each section, and the mean of intensities of three sections was considered as the mean of ILT3 staining intensity in normal or tumor-bearing ovaries.

### One-dimensional Western Blot

The expression of ILT3 proteins by normal ovaries or ovarian tumors as well as OSE from normal ovaries or ovaries with tumor was determined by Western blot analysis using primary and secondary antibodies mentioned above. On the basis of immunohistochemical staining results, representative samples of ovarian as well as OSE homogenates from normal or ovarian tumors at early and late stages were used in immunoblot analysis. Immunoreactions on the membrane were visualized as a chemiluminescence product (Super Dura West substrate; Pierce/Thermo Fisher, Rockford, IL), and the image was captured using a Chemidoc XRS (Bio-Rad, Hercules, CA). Digital images obtained with Chemidoc XRS were analyzed by Quantity One software (Bio-Rad) according to the manufacturer's recommendation, and the intensities of immunoreactive bands were expressed as density per intensity in squared millimeter and the mean of intensities for each normal or pathologic group as well as for the stages of OVCA were calculated. No immune reaction was observed in controls where protein samples were omitted. Serum samples for Western blot analysis were selected similar to ovarian samples and tested to confirm the presence of soluble ILT3.

### Reverse Transcription–Polymerase Chain Reaction

ILT3 messenger RNA (mRNA) expression in ovarian tissues or epithelial cells from normal hens or hens with OVCA was assessed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) as reported previously [29]. Representative samples of normal ovaries or ovaries with tumor as well as OSE from hens were selected for RT-PCR analyses based on their immunoreactivities in immunohistochemistry and immunoblot analysis were used. Hen-specific ILT3 primers were designed by Oligoperfect Designer software (Invitrogen, Carlsbad, CA) using the ILT3 sequence from the National Center for Biotechnology Information (GenBank: NM\_001146134.1). The forward primer was 5-TGG CTG TAC CAG GAA AGA GG and the reverse primer was 5-CTC TGA TGC CCC TAC TGA CC.  $\beta$ -Actin was used as the endogenous control with a forward primer of

TGCGTGACATCAAGGAGAAG and a reverse primer of ATGC-CAGGGTACATTGTGGT. The expected base pair size for the ILT3 amplicon was 150 bp and that for  $\beta$ -actin was 300 bp. PCR amplicons were visualized in a 3% agarose gel (Pierce) in TAE buffer and stained with ethidium bromide, and images were captured using a ChemiDoc XRS system (Bio-Rad). PCR products were sequenced, and the sequence was the same as the sequence of primers from the National Center for Biotechnology Information GenBank (NM\_001146134.1).

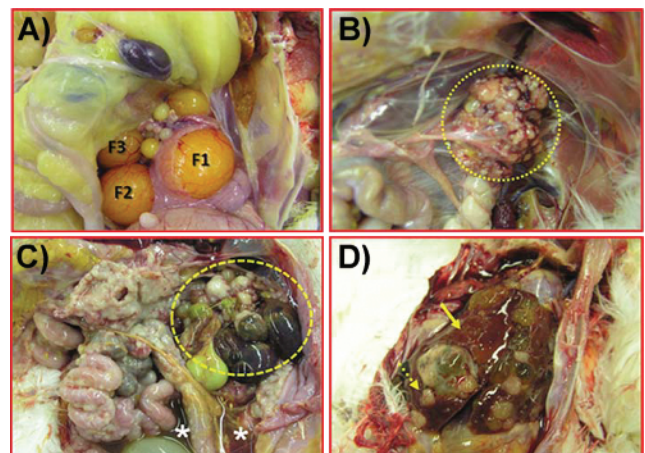
### Statistical Analysis

The differences in the pixel intensities of ILT3 immunostaining among different histologic subtypes and stages (early *vs* late) were assessed by two-way analysis of variance. This was followed by pairwise comparison between the histologic subtypes (normal, endometrioid, mucinous, and serous) within each stage and comparison of the stages within histologic subtypes by two-sample *t* tests and alternative Mann-Whitney tests. All reported *P* values are 2-sided, and *P* < .05 was considered significant. Statistical analyses were performed with SPSS (PASW) version 18 software (IBM, Inc, Armonk, NY) and R statistical software.

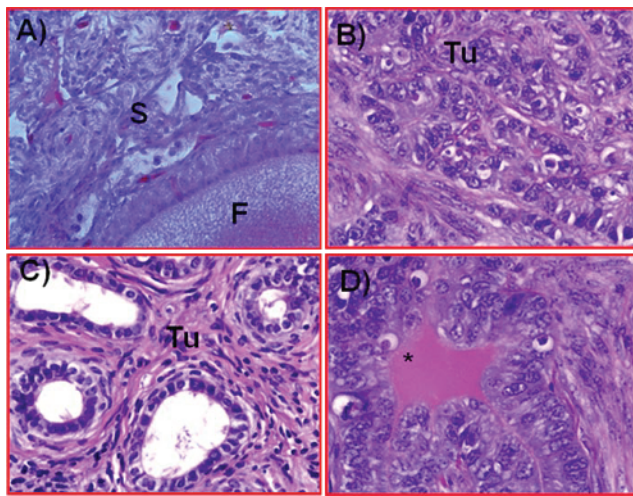
## Results

### Morphologic and Histologic Features of Hen Ovaries and Ovarian Tumors

**Gross morphology.** A fully functional ovary in a healthy laying hen contained five to six developing large preovulatory follicles (Figure 1A), whereas the ovaries of low-laying healthy hens contained less than three preovulatory follicles. In normal hens that had stopped egg laying, ovaries and oviducts were regressed without any detectable abnormality. Solid tissue masses either limited to a small part or to the whole ovary and with or without ascites were observed in 14 hens and



**Figure 1.** Gross morphology of normal ovaries and ovaries with tumor in laying hens. (A) Normal ovary: A normal laying hen ovary contained a hierarchy of multiple large preovulatory follicles (F1-F3) with an active oviduct. (B) OVCA at early stage in a laying hen. The tumor mass was limited to the ovary (shown in a dotted circle). (C and D) OVCA at late stages in laying hens. Tumors were metastasized to distant organs including intestine (C) and liver (D, arrows indicate the examples of tumor seeding) with accompanied profuse ascites (\*).



**Figure 2.** Histologic presentation of different types of malignant ovarian tumors in laying hens detected in the present study. Formalin-fixed paraffin-embedded ovarian tissues were stained for hematoxylin and eosin. (A) A section of a normal ovary showing a follicle embedded in the ovarian stroma. (B) A section of an ovarian serous carcinoma-containing tumor cells with large pleomorphic nucleus and mitotic nuclear bodies. (C) A section of an ovarian endometrioid carcinoma showing confluent back-to-back tumor glands containing a single layer of epithelium with sharp luminal margin. (D) A section of an ovarian mucinous carcinoma. The tumor contained a single layer of columnar epithelium with intercalated ciliated goblet cells. Mucin-like secretion is seen in the lumen of the tumor gland (\*). Original magnifications:  $\times 40$ . F indicates follicle; S, stroma; Tu, tumor.

classified as hens with early-stage OVCA (Figure 1B). In 17 hens, the tumor had metastasized to abdominal organs with moderate to profuse ascites and classified as hens with late-stage OVCA (Figure 1, C and D).

**Histopathology.** A total of 105 hens were found to have normal ovaries in which embedded primordial and primary follicles were observed in the ovarian stromal tissue (Figure 2A). Ovarian tumors were confirmed by routine histology in 31 hens that had solid masses limited to the ovaries ( $n = 14$ , early stage) or metastasized to other organs ( $n = 17$ , late stage). In addition, microscopic OVCA were detected in 12 hens without any grossly detectable solid mass in the ovary and grouped in early-stage OVCA. Thus, a total of 26 ( $14 + 12$ ) hens had early and 17 had late-stage OVCA. Tumors were typed (Figure 2, B-D) as serous ( $n = 18$ ), endometrioid ( $n = 13$ ), mucinous ( $n = 10$ ), as well as mixed ( $n = 2$ , seromucinous and endoserous) as reported previously [22].

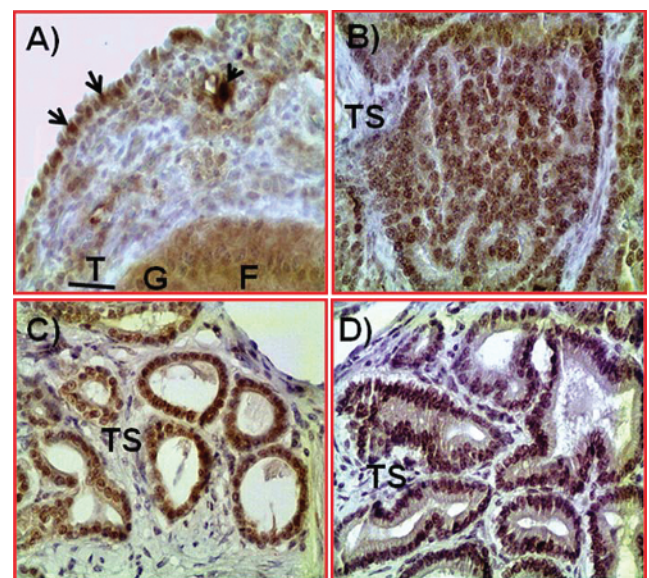
### Tissue Expression of ILT3

**Immunohistochemical detection of ovarian ILT3 expression.** T-lymphocyte-like rounded cells and macrophage-like irregular-shaped cells in the stroma of normal ovaries or ovaries with tumor were found positive for ILT3. Some surface epithelial cells (not all) above the developing cortical follicles in normal ovaries and the epithelium of the ovarian tumors were also stained positive for ILT3 expression (Figure 3, A-D). The expression of ILT3 by the epithelial cells of the tumor in hens with OVCA varied with respect to the histologic subtypes of tumors and their stages. As compared with normal hens (mean  $\pm$  SD =  $23029.23 \pm 2725.01$ ), the intensities of ILT3 expression

were significantly higher ( $P < .001$ ) in hens with early-stage OVCA irrespective of their histologic subtypes (Figure 4). Similar patterns were also observed for hens with late-stage OVCA.

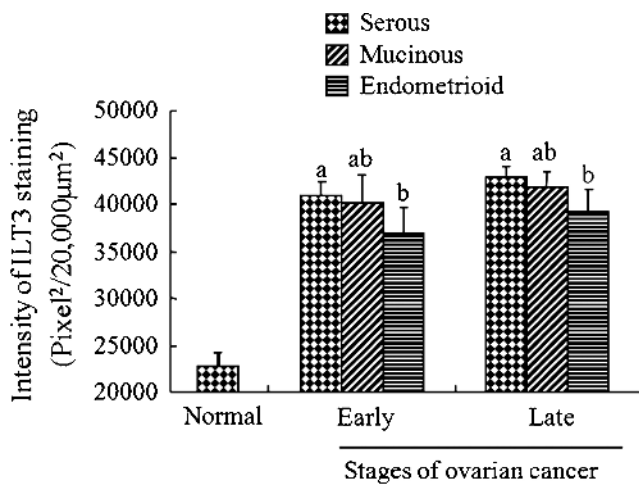
Among different histologic subtypes at early-stage OVCA, the intensities of ILT3 staining were lowest in hens with ovarian endometrioid tumors (mean  $\pm$  SD =  $36,807.56 \pm 2843.56$ ) followed by mucinous ( $40,207.86 \pm 2858.27$ ) and highest in serous OVCA ( $40,924.40 \pm 1400.26$ ) in a  $20,000\text{-}\mu\text{m}^2$  area of tissue (Figure 4). The differences in ILT3 staining intensities were significantly higher in hens with serous OVCA than hens with endometrioid OVCA irrespective of their stages ( $P < .028$  and  $.025$  for early and late stages of OVCA, respectively). However, significant differences in ILT3 staining intensities were not observed between the hens with ovarian endometrioid and mucinous OVCA as well as mucinous and serous OVCA at early and late stages. In hens with serous OVCA, the intensities of ILT3 staining were significantly high in late stages than in early stage ( $P < .05$ ). However, a significant difference in ILT3 staining intensities between the early and late stages of hens with ovarian endometrioid or mucinous OVCA was not observed.

**Immunoblot analysis for ILT3 protein in ovarian tissues and serum samples.** Immunohistochemical observation of ILT3 expression was confirmed by immunoblot analysis using homogenates of normal ovaries and ovaries with tumors as well as OSE from normal ovaries and ovarian tumors. As expected, immunoreactive ILT3 protein with a band size of approximately 55 kDa was detected in the homogenates of OSE and ovarian tissues from normal hens and hens with OVCA at early stage (Figure 5A). Similar patterns were also observed for serum



**Figure 3.** Expression of ILT3 by normal ovaries or ovaries with tumor in laying hens. (A) A section of normal ovary showing ILT3+ OSE cells (examples are shown by arrows) near a developing follicle. An immune cell-like ILT3+ cell is seen beneath the ovarian surface (arrowhead). (B-D) Expression of ILT3 by the epithelium of the ovarian serous (B), endometrioid (C), and mucinous (D) carcinomas at late stages in laying hens. Intense staining for ILT3 by epithelial cells of the tumors was observed in all tumor types. F indicates follicle; G, granulosa layer; T, theca layer; TS, tumor stroma.





**Figure 4.** Changes in the ovarian ILT3 expression are associated with ovarian tumor development and progression. The intensity of ILT3 staining is expressed as pixel values (mean  $\pm$  SD) in a 20,000- $\mu\text{m}^2$  area of the ovarian stroma in normal ovaries or ovaries with tumor. Compared with normal hens, the intensities of the ILT3 staining were significantly greater in hens with early-stage OVCA ( $P < .001$ ) and increased further in hens with late-stage OVCA irrespective of their tumor types. The intensity of ILT3 staining was significantly lower ( $P < .03$ ) in hens with endometrioid OVCA than hens with serous OVCA. Significant differences were not observed in the intensity of ILT3 staining between the hens with serous and mucinous OVCA irrespective of their stages. Bars with different letters within the same group indicate significant differences in ILT3 staining intensity.

samples (data not shown). Compared with the normal OSE or OSE from ovarian tumors at early stage, ILT3 protein expression was stronger in the OSE from hens at late-stage OVCA (Figure 5A). Conversely, the expression of immunoreactive ILT3 proteins was weaker in the homogenates of ovarian endometrioid tumors, moderate in mucinous, and stronger in ovarian serous tumors at the early stage (Figure 5A). Similar patterns were also observed for ovarian tumors at late stage (data not shown). These results confirm the immunohistochemical observation that epithelial cells of ovarian tumors in hens express ILT3.

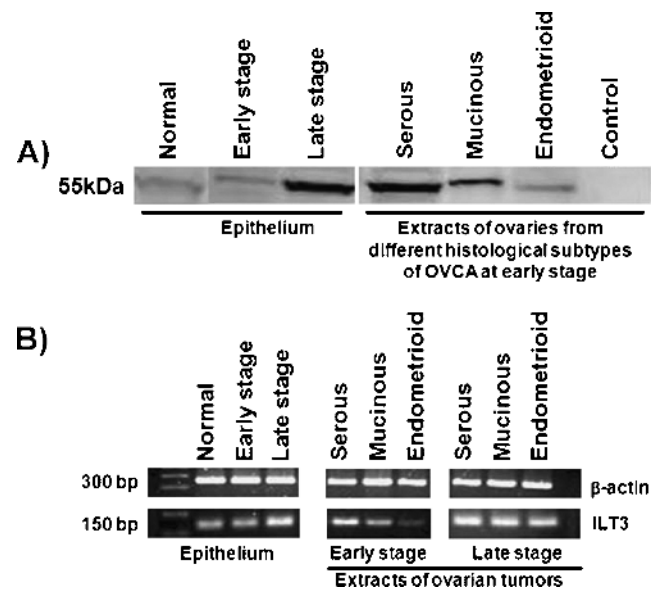
**Expression of ILT3 mRNA.** Observed variations in ILT3 protein expression among different histologic subtypes of OVCA and their stages were confirmed by ILT3 mRNA expression. Hens with early serous and mucinous ovarian tumors showed strong amplification signal for ILT3, whereas its amplification was low for endometrioid ovarian tumors. However, no differences were observed for ILT3 mRNA expression by different histologic subtypes of late-stage OVCA (Figure 5B). Similar patterns were also observed for the epithelial cells of ovarian tumors. Thus, the results of gene expression study confirm the immunohistochemical and immunoblot analysis observations that the epithelium of ovarian tumors expresses ILT3 proteins.

## Discussion

This is the first report on the expression of ILT3 by the epithelium of ovarian tumors in laying hens, an animal model of spontaneous OVCA. This study showed that the expression of ILT3 increases significantly in association with ovarian tumor development and progression. These results suggest that laying hens can be used to generate information on the mechanism of spontaneous ovarian tumor-associated immuno-

suppression and may lead to the development of antitumor immunotherapies and the testing of their efficacies.

Recent progresses in the understanding of tumor-immune interactions have led to the successful development of a number of immunotherapeutic approaches. However, tumor escape from immune recognition is a significant barrier to the success of these immunotherapies. Although the process of escaping immune surveillance by tumors or suppression of antitumor immune response is not well understood, tumor cells, immune cells, and other stromal cells in the tumor surroundings have been reported to interact and create an immunosuppressive microenvironment through a variety of immunosuppressive factors [30]. Enhanced expression of ILT3 by few members of the immune system has been suggested as one of such immunosuppressive factors in the tumor microenvironment [31]. In the present study, in addition to immune cell-like cells, the epithelium of the ovarian tumors in laying hens also



**Figure 5.** Examples of immunoreactive ILT3 protein (A) or mRNA (B) expression in the homogenates of OSE and ovaries from normal hens or hens with OVCA by one-dimensional Western blot (WB) analysis and semiquantitative PCR, respectively. One-dimensional WB (A): Immunoreactive ILT3 protein of approximately 55-kDa molecular weight was detected in the 1) OSE extract from normal or ovarian tumors and 2) homogenates from different histologic subtypes of ovarian tumors at early stage of OVCA. Compared with the normal OSE or OSE from ovarian tumors at early stage, ILT3 protein expression was stronger in the OSE from hens at late-stage OVCA. Serous ovarian tumors at the early stage showed strong ILT3 staining followed by mucinous and endometrioid tumors. No ILT3 expression was detected in negative control in which protein sample was omitted. RT-PCR (B): mRNA expression by the OSE extract from normal or ovarian tumor and from different histologic subtypes of ovarian tumors at early and late stages of OVCA. Compared to the normal OSE, or OSE from ovarian tumor at early stage, a strong expression of ILT3 mRNA was observed in the OSE from hens with late-stage OVCA. Similarly, strong expression patterns were also observed for the homogenates of ovarian tumors at late stages than early stages irrespective of tumor subtypes. As observed in immunohistochemistry and immunoblot analysis, endometrioid tumors at early stage were weaker in ILT3 mRNA expression than their counterpart serous and mucinous tumors. No ILT3 mRNA expression was detected in the negative control in which mRNA sample was omitted.

expressed ILT3. Under normal physiological condition, ILT3 has been reported to be expressed selectively by professional antigen-presenting cells including monocytes, macrophages, and dendritic cells [32]. The expression of ILT3 on exposure to alloantigen-specific suppressor T cells or cytokines by nonprofessional antigen-presenting cells, like endothelial cells as well as tumor cells from chronic lymphocytic leukemia, was also reported previously [33,34]. Thus, the present results suggest that ovarian tumors also express immunosuppressive ILT3 as reported for few other cancers.

In the present study, compared with normal hens, the intensity of the ILT3 expression was significantly high in hens with early-stage OVCA and increased further as the disease progressed to late stages, suggesting that changes in ILT3 expression may be associated with ovarian tumor development and progression. The presence of active antitumor immune responses against ovarian tumors at the early stage has been reported earlier [9]. However, despite the presence of an antitumor immune response, OVCA in most cases, progress to late stages. Although the precise mechanisms of such immune escaping are not known, our results suggest that the enhanced expression of immune inhibitory receptor ILT3 by the ovarian tumors may contribute to the progression of OVCA.

The results of the present study suggest that the expression pattern of ILT3 varies with the different histologic subtypes of ovarian tumors. Significantly high ILT3 staining intensity was observed in serous compared with endometrioid ovarian tumors at early as well as late stages of OVCA. The specific reasons for higher ILT3 expression by serous OVCA are not known. It is possible that higher ILT3 expression will contribute to the faster progression of OVCA by imparting higher immune tolerance to tumors. Serous malignant ovarian tumors are considered aggressive tumors, and increased ILT3 expression may contribute to their faster progression. Similar observations in cancers of other organs were also reported previously [31,34]. Thus, it is possible that ILT3 will contribute to ovarian tumor progression by suppressing antitumor immunity.

In the present study, a portion of the epithelial cells of the normal ovarian surface epithelium above the cortical developing follicles (not all epithelial cells) were positive for ILT3 expression albeit with lower intensity than the epithelium of ovarian tumors. Although the precise reason(s) for such expression is not known, it is possible that ILT3 expressed by surface epithelial cells near cortical follicles will protect them from immune cells by suppression of local antifollicular autoimmune response. Various structures in the developing follicles including the perivitelline membrane, granulosa and theca layers, as well as the degenerated cellular components of postovulatory follicles may appear as "foreign" to the circulating immune cells because these structures were not present during the evolution and maturation of the immune system.

Our understanding regarding the biology and the role of ILT3 in the context of OVCA is very limited, in part, because of the lack of an animal model that develops spontaneous OVCA. Because of the difficulty of detecting OVCA at an early stage, access to patient specimens to study and develop an effective antitumor immunotherapy is difficult and time consuming. Similarities between the spontaneous OVCA in humans and hens in histologic subtypes, risk factors (e.g., incessant ovulation), and expression of several molecular makers of OVCA represent a high probability that results obtained from this study may be translated to clinics. Furthermore, one of the most important advantages of this animal model relative to the possibilities of translating current findings to OVCA in humans is the ability to

monitor hens by contrast enhanced ultrasound imaging using equipment and mechanical setting similar to those used in clinics [25,26]. Thus, there will be less variation in imaging parameters between hens and humans because of the use of similar equipment. With the advancement in *in vivo* imaging technology, ligands that bind to the epithelial cells of the ovarian tumor are being developed to use as anti-tumor therapies, and their effectiveness in tumor ablation can be monitored in this animal model using contrast-enhanced ultrasound scanning [35]. Because the epithelial cells of the tumor in OVCA hens express ILT3, laying hens may be used to test the efficacy of anti-ILT3 drug by molecular-targeted ultrasound imaging of ovarian tissues. Thus, it will bring a significant change in the development of tumor specific therapeutics specially immunoenhancing drugs and lead to the development of treatment modalities for OVCA patients. Taken together, information on ILT3 expression and its association with OVCA progression will aid in the development of anti-ILT3 immunotherapies, which will lay the foundation for clinical studies and may ultimately lead to the development of effective therapies for the treatment of OVCA.

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## Use of contrast-enhanced ultrasound imaging with microbubbles targeted to $\alpha_v\beta_3$ integrins to enhance detection of early-stage ovarian tumors.

**Sub-category:**

Ovarian Cancer

**Category:**

Gynecologic Cancer

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Abstracts that were granted an exception in accordance with ASCO's Conflict of Interest Policy are designated with a caret symbol (^) here and in the printed Proceedings.

**Abstract Disclosures****Abstract:**

**Background:** Lack of an early detection test makes ovarian cancer (OVCA) a lethal gynecological malignancy. Tumor associated neo-angiogenesis (TAN) is an early event in tumor development and represents a potential target for early detection. Traditional transvaginal ultrasound (TVUS) cannot detect TAN at early stage. The goal of this pilot study was to examine the enhancement of TVUS using  $\alpha_v\beta_3$  integrins targeted microbubble ultrasound contrast agent (UCA) in laying hens, a preclinical spontaneous model of human OVCA. **Methods:** 3-4 years old hens (n=50) were selected randomly and scanned continuously by TVUS before, during and after UCA (Visister-integrins, Targeson Inc. CA) injection at brachial veins. UCA was visualized using a low mechanical index contrast imaging pulse sequence. All pre- and post-contrast injection images were archived and analyzed off-line. Gross diagnosis was recorded at euthanasia and tissues were processed for histology. Tumors and their types were confirmed by routine histology. TAN markers (SMA and  $\alpha_v\beta_3$  integrins) were detected by immunohistochemistry. TAN vessels were counted and correlation with ultrasound prediction was examined. **Results:**  $\alpha_v\beta_3$ -integrins targeted UCA enhanced the visualization of ovarian tumors significantly in laying hens. At gray scale, UCA bounded areas appeared as a ring on the ovarian surfaces of 8 of 50 hens and were suspected for ovarian tumors. Tumors were confirmed in all hens predicted to have OVCA. In 7 hens tumors were early stage and in one hen the tumor metastasized to the oviduct. A microscopic lesion was found in one hen which was not detected by ultrasound imaging. Thus 9 of 50 hens had ovarian tumors and UCA detected approximately 88% at early stages. The frequency of immunopositive TAN vessels (SMA and  $\alpha_v\beta_3$  integrins positive) were significantly higher in OVCA hens than normal hens ( $P<0.05$ ) and was positively correlated (0.86,  $P<0.05$ ) with ultrasound prediction. **Conclusions:** Our results demonstrate that UCA targeting  $\alpha_v\beta_3$  integrin enhanced TVUS detection of early stage ovarian tumors in a pre-clinical model and may form the foundation for a clinical study. Support: Department of Defense (#09-3303), Sramek Foundation.

**► Associated Presentation(s):**

1. Use of contrast-enhanced ultrasound imaging with microbubbles targeted to  $\alpha_v\beta_3$  integrins to enhance detection of early-stage ovarian tumors.

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## Presentation Abstract

Abstract Number: 3190

Presentation Title: Interleukin (IL-16) and tumor associated neo-angiogenesis detects ovarian cancer at early stage

Presentation Time: Tuesday, Apr 05, 2011, 8:00 AM -12:00 PM

Location: Exhibit Hall A4-C, Poster Section 13

Poster Section: 13

Poster Board Number: 27

Author Block: Aparna Yellapa<sup>1</sup>, Jacques S. Abramowicz<sup>1</sup>, Pincas Bitterman<sup>1</sup>, Janice M. Bahr<sup>2</sup>, Michael J. Bradaric<sup>1</sup>, Seby L. Edassery<sup>1</sup>, Sameer Sharma<sup>1</sup>, Animesh Barua<sup>1</sup>. <sup>1</sup>Rush University Medical Center, Chicago, IL; <sup>2</sup>University of Illinois at Urbana-Champaign, Champaign, IL

Abstract Body: **Background:** The lack of an early detection test is one of the reasons of high mortality rate of women due to epithelial ovarian cancer (OVCA). Understanding the pathogenesis and progression of OVCA is essential to establish an effective early detection test. Tumor associated -immuno-chemotaxis (TAI) and -neoangiogenesis (TAN) are the two earlier events in tumor development. CD8+ T cells in tumor vicinity produce immune-chemotactic IL-16 cytokines which stimulates the production of pro-angiogenic cytokines responsible for TAN. Ovarian TAI and TAN represent potential target for an effective early detection test. Access to patients at early stage OVCA is very difficult and laying hens develop spontaneous OVCA with histopathology similar to humans. **Objectives:** The goal of the present study was to determine the feasibility of markers of ovarian TAI and TAN in detecting early stage OVCA in laying hens.

**Materials and Methods:** 3 years old laying hens (normal, low or stopped egg-laying) were scanned by Ultrasound, sera were collected, hens were euthanized and ovarian tissues were processed for paraffin and frozen sections, and mRNA extraction. Ovarian tumor stages were confirmed at gross and routine histology. Samples were divided into 4 groups namely normal (control), early stage OVCA [microscopic or tumors limited to the ovaries], late stage OVCA, with non-tumor ovarian abnormalities (atrophied ovaries). Sera were tested for IL-16 levels by ELISA and confirmed by 2D-Western blot (WB). Ovarian expression of TAN markerS (VEGF) and, neoangiogenic microvessel density (MVD) as well as IL-16 mRNA was determined.

**Results:** The population of CD8+ T cells and the serum IL-16 levels were significantly higher in tumor hens ( $P < 0.05$ ) than normal hens. Significant increase in IL-16 levels in hens with microscopic tumor (undetectable at gross examination) suggesting that serum IL-16 may be a potential indicator of ovarian tumors at very early stage. Differences in serum IL-16 levels were not observed between hens with non-tumor ovarian pathology and normal hens suggesting that increased serum IL-16 levels in OVCA hens are tumor specific. Two immunoreactive bands (12 & 50kDa) for IL-16 were identified in the ovary while only one band (50 kDa) was detected in sera suggesting that IL-16 may be the active in tetramerized form. IL-16 protein and mRNA expression as well as the frequency of MVD were significantly higher in hens with OVCA than healthy hens. VEGF expressing microvessels were localized in the ovarian stroma preceding the tumor indicating that ovarian TAN precedes tumor progression.

**Conclusion:** The results of the study suggest that changes in serum levels of IL-16 are positively correlated with tumor initiation and progression. Thus serum IL-16 level together with marker of ovarian TAN may constitute a feasible test for the early detection of OVCA. Support: Prevent Cancer Foundation, Sramak Foundation and DOD (OC#093303).

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## Poster Presentations - Biomarkers of Ovarian, Prostate, and Bladder Cancers

### Abstract 3646: Expression of death receptor 6 (DR6) increases in association with ovarian tumor development and progression

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**Background:** Because of the lack of an early detection test, ovarian cancer (OVCA) remains a lethal malignancy of women. OVCA differs from other malignancies in its specific dissemination pattern that the tumor typically spreads in a diffuse intra-pelvic and abdominal manner. Thus tumor microenvironment plays an important role in tumor dissemination and suppression of anti-tumor immunity is a hallmark of tumor progression. Although the precise mechanism of immune evasion by the tumor is not known, expression of death receptor 6 (DR6) has been suggested to be associated with tumor progression in several malignancies. DR6 is a member of the TNF receptor superfamily and a regulator of immune function. Expression of DR6 by ovarian tumors is unknown. Access to patients at early stage OVCA is very difficult. Laying hens develop OVCA spontaneously with histopathology similar to humans. **Objectives:** The goal of the present study was to determine whether DR6 is expressed by ovarian tumors at early stage and to examine whether DR6 expression is associated with OVCA tumor progression in the laying hen model of human OVCA. **Materials and Methods:** 3 years old normal, low or stopped egg-laying hens were scanned by ultrasound, sera were collected, hens were euthanized and ovarian tissues were processed for paraffin and frozen sections, immunoblotting and mRNA extraction. Stages of OVCA were confirmed at gross morphology and routine histology. Samples were divided into 3 groups: normal (control), early and late stages of OVCA. Sera were tested for DR6 levels by ELISA and confirmed by 2D-Western blot (WB). Expression of DR6 by normal and tumor ovaries was examined by immunohistochemistry (IHC), WB and RT-PCR. **Results:** DR6 was expressed by the epithelium of the ovarian tumor and angiogenic microvessels in the tumor stroma. The population of DR6+ microvessels and serum levels of DR6 was significantly higher in hens with ovarian tumors than the normal hens. Immunoblotting and gene expression analysis confirmed IHC observations. There was a significant increase in DR6 expression in hens with early stage OVCA (including microscopic tumor) suggesting that serum DR6 may be a potential indicator of ovarian tumors at very early stage. The frequencies of DR6+ cells were significantly higher in hens with late stage OVCA than early stage suggesting that DR6 may be involved in ovarian tumor development and progression in laying hens. **Conclusion:** For the first time, this study showed that epithelial cells in ovarian tumors express DR6 and results suggest that changes in serum levels as well as tissue expression of DR6 are associated with tumor initiation and progression. Thus serum DR6 may be useful as a surrogate maker of OVCA, target for anti-tumor immunotherapy as well as molecular imaging. This study will provide a foundation for a clinical study. **Support:** DOD (OC#093303), NCI-POCRC (P50 CA83636) and the Elmer Sylvia and Sramek Foundation.

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## Poster Presentations - Tumor Immunobiology 3

### **Abstract 5415: Expression of immunosuppressive leukocyte inhibitory immunoglobulin like transcript 3 (ILT3) receptors increases with the development and progression of ovarian tumors**

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**Background:** Immunosuppressive tumor microenvironment has been suggested as one of the barriers to anti-tumor immune response in patient with ovarian cancer (OVCA). Leukocyte Inhibitory Receptor Immunoglobulin-like Transcript 3 (ILT3) has been proposed as a factor involved in the suppression of immune response in several malignancies. However, the expression and role of ILT3 in the development and progression of ovarian tumors has not yet been studied. **Objective:** The goal of this study was to examine the expression and association of ILT3 in ovarian tumors in laying hen, a preclinical model of OVCA in humans. **Methods:** A cohort of mature White Leghorn laying hens with normal or low egg laying rates were evaluated by transvaginal ultrasound scan (15 normal hens and 30 hens with OVCA). All hens were euthanized and serum and ovarian tissues from all hens were collected and processed for immunohistochemistry (IHC), Western blotting (WB) and RT-PCR. Presence of tumors and tumor staging were confirmed by gross morphology and histology. Expression of ILT3 was examined by IHC, WB and RT-PCR. **Results:** Immune cell like cells in the ovarian stroma as well as epithelium of the normal ovaries and ovarian tumors expressed ILT3. The intensity of ILT3 expression was significantly higher in hens with OVCA than normal hens. Moreover, the intensity of ILT3 expression varied with histological subtypes of ovarian tumors and was significantly higher in serous OVCA than endometrioid OVCA. A band of approximately 55kDa of ILT3 protein was detected in the homogenates of normal ovarian surface epithelium or tumor epithelium as well as whole normal or ovarian tumor extracts. Similar to IHC observations, protein expression was stronger in hens with OVCA than in normal hens. **Conclusion:** This is the first study to show that immunosuppressive ILT3 is expressed by ovarian tumor epithelium and the intensity of expression increases as the disease progresses. Thus the results are consistent with the hypothesis that ILT3 may be involved in the suppression of anti-tumor immunity in OVCA. The laying hens may be a useful model to understand the OVCA-associated expression of ILT3. This animal model also offers the opportunity to develop and test anti-ILT3 therapy to enhance anti-tumor immunity against OVCA in humans. **Support:** Department of Defense (OC#93303) and Elmer Sylvia and Sramek Foundation.

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**Oral Presentations  
May 10, 2012  
7pm Main Conference Room**

**Molecular targeted ultrasound imaging of avb3-integrins expressing microvessels in association with anti-NMP antibodies detects ovarian cancer at early stage**

Animesh Barua

Departments of Pharmacology, Pathology and Obstetrics & Gynecology, Rush University, Chicago

**Ovarian Cancer Survivors' Experiences of Self-Advocacy: A Focus Group Study**

Teresa Hagan

University of Pittsburgh School of Nursing, Pittsburgh, PA

**Sex-steroid hormones and epithelial ovarian cancer: a nested case-control study**

Annekatriin Lukanova

Division of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany

**Potential effect of the Risk of Ovarian Cancer Algorithm (ROCA) on the mortality outcome of the Prostate, Lung, Colorectal and Ovarian (PLCO) Trial**

Paul Pinsky

Division of Cancer Prevention, NCI

**Monoclonal antibody-based immunotherapy of ovarian cancer: targeting of differentiated and cancer initiating cells with the B7-H3-specific mAb 376.96 and sunitinib**

Donald Buchsbaum

Department of Radiation Oncology, University of Alabama at Birmingham

**Meso-TR3: A Novel TRAIL-Based Targeted Therapeutic in Ovarian Cancer**

Gunjal Garg

Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis

**PGE2 in ovarian cancer-associated immune dysfunction**

Pawel Kalinski

University of Pittsburgh Cancer Institute, Pittsburgh, PA

**A genetically engineered mouse model for high grade serous "ovarian" carcinoma arising in the fallopian tube.**

Ruth Perets

Department of Medical Oncology, Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA

## Molecular targeted ultrasound imaging of $\alpha_v\beta_3$ -integrins expressing microvessels in association with anti-NMP antibodies detects ovarian cancer at early stage

**Background:** Changes in nuclear morphology including nuclear matrix proteins (NMPs) followed by tumor associated neoangiogenesis (TAN) are the two earlier events in malignant transformation and progression of ovarian cancer (OVCA). Anti-NMP antibodies are produced in response to NMPs shed during malignant transformation. Expression of  $\alpha_v\beta_3$ -integrins by TAN vessels is one of the features of ovarian TAN. Anti-NMP antibodies and ovarian TAN may represent markers of early stage OVCA. Identification of patients at early stage OVCA is very difficult and laying hens have been shown to develop spontaneous OVCA with histopathology similar to humans. The goal of this study was to examine the feasibility of  $\alpha_v\beta_3$ -integrin targeted transvaginal ultrasound (TVUS) imaging and anti-NMP antibodies in detecting OVCA at early stage.

**Methods:** Hens with (n= 50) or without (n= 20) serum anti-NMP antibodies and without any TVUS detectable ovarian abnormality were selected for prospective monitoring by  $\alpha_v\beta_3$ -integrins targeted TVUS at 15 weeks interval up to 45 weeks. Hens were scanned before, during and after injection of targeted microbubbles at each interval. Pre- and post-injection images archived and analyzed off-line. Hens were euthanized at diagnosis for OVCA or at the end of the 45 weeks. Gross diagnosis was recorded at euthanasia. Serum and tissues were processed for histology, immunohistochemistry (SMA and  $\alpha_v\beta_3$  integrins) and immunoblotting.

**Results:** Within 30-45 weeks of monitoring, 7 hens with serum anti-NMP antibodies developed OVCA spontaneously.  $\alpha_v\beta_3$ -integrins targeted microbubbles enhanced the visualization of ovarian tumors remarkably. Targeted microbubbles bounded areas appeared as a ring on the ovarian surfaces of 5 hens on TVUS and suspected for OVCA. Tumors were confirmed in all hens predicted to have OVCA by targeted TVUS at euthanasia. In 4 hens, tumors were limited to the ovaries (early stage) and in one hen the tumor metastasized to abdominal cavity. Targeted TVUS imaging could not detect two hens with microscopic lesions without any solid tumor mass. Thus 7 of 50 hens had OVCA and targeted microbubbles detected approximately 72% (5 of 7 hens). Serum reacted against NMP antigens of various molecular wt from malignant ovaries in immunoblotting. The frequency of TAN vessels (SMA and  $\alpha_v\beta_3$ -integrins expressing) were significantly higher in OVCA hens than normal hens ( $P<0.05$ ).

**Conclusions:** Targeted imaging enhanced TVUS detection of early stage OVCA. Anti-NMP antibodies together with targeted imaging may constitute an early detection test for OVCA. The results may form the foundation for a clinical study.

Support: Department of Defense (#09-3303), Sramek Foundation.

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## Presentation Abstract

Abstract  
Number: 3471

Presentation  
Title: **Anti-NMP autoantibodies together with serum levels of death receptor 6 (DR6) detect ovarian tumors at early stage**

Presentation  
Time: Tuesday, Apr 09, 2013, 1:00 PM - 5:00 PM

Location: Hall A-C, Poster Section 1

Poster Board  
Number: 17

Author  
Block: Animesh Barua<sup>1</sup>, Jacques S. Abramowicz<sup>1</sup>, Janice M. Bahr<sup>2</sup>, Salvatore A. Grasso<sup>1</sup>, Sameer Sharma<sup>1</sup>, Sanjib Basu<sup>1</sup>, Jacob Rotmensch<sup>1</sup>, Pincas Bitterman<sup>1</sup>. <sup>1</sup>Rush Univ. Medical Ctr., Chicago, IL; <sup>2</sup>Univeristy of Illinois at Urbana-Champaign, Urbana-Champaign, IL

Abstract  
Body: **Background:** High rate mortality in patients with ovarian cancer (OVCA) is attributed to the lack of an effective early detection test. OVCA progression differs from other malignancies in that the tumor typically spreads in a diffuse intra-abdominal manner. Thus tumor microenvironment plays an important role both in the inhibition, as well as progression of ovarian cancer. Malignant nuclear transformation is an early step in OVCA development which sheds nuclear matrix proteins (NMP) in circulation. The immune system responds to secreted NMPs by producing anti-NMP antibodies. Conversely, the tumor evolves immunosuppressive mechanisms to escape anti-tumor immunity. Death receptor 6 (DR6) has been suggested as an immunosuppressive agent expressed in several malignancies including OVCA. Thus, serum anti-NMP antibodies and DR6 may represent early markers of OVCA. However, association of anti-NMP antibodies and DR6 expression during OVCA development is unknown.

**Objectives:** The goal of this study was to determine if serum anti-NMP antibodies and DR6 levels are associated with malignant ovarian transformation and to examine their feasibility in detecting OVCA at early stage.

**Materials and Methods:** Access to OVCA patients at early stage is very difficult. We used laying hen model of spontaneous OVCA. 3-4 years old normal, low or stopped egg-laying hens were selected by ultrasound scanning, sera were collected, hens were euthanized and tissues were processed for histology, immunohistochemistry (IHC) and RT-PCR. Sera were processed for ELISA for DR6 and anti-NMP antibodies. Immunoreactions were confirmed by immunoproteomics. Tumor stage and their histological types were determined by gross inspection and routine histology. Samples were divided into 3 groups namely, normal (n= 20), early (n=15) and late (n = 17) stages of OVCA.

**Results:** Prevalence of anti-NMP antibodies was detected in approx. 75% hens at early stage and 94% hens at late stage OVCA. All serum samples from tumor hens detected NMP antigens of 30-80kDa in immunoproteomic studies. The expression of DR6 by ovarian malignant epithelium was significantly (p<0.001) higher in hens with early stage OVCA than normal hens and increased further in hens with late stage OVCA. Similar patterns were observed in immunoblotting and gene expression studies. Serum levels of DR6 were significantly (p<0.001) higher in hens with early and late stages of OVCA. Increase in serum DR6 levels were positively correlated with the prevalence of serum anti-NMP antibodies.

**Conclusion:** Results of this study suggest a concurrent prevalence of immune responses and immunosuppression to ovarian tumors. Anti-NMP antibodies and DR6 are associated with tumor initiation and progression. Thus, serum anti-NMP antibodies together with DR6 levels may detect OVCA at early stage. This study will provide a foundation for clinical study. Support: DOD Award # W81XWH-10-1-0523

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